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Novel Small Molecules Disabling the IL-6/IL-6R/GP130 Heterohexamer Complex

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14. ABSTRACT

There is an inherent connection between cancer and chronic inflammation. One of the key inflammatory cytokines, IL-6, is a strong biomarker and therapeutic target of breast tumor progression, especially for late-stage, metastatic and resistant breast cancers. This project is to develop "first-in-class" small molecule IL-6 inhibitors for potential novel drug discovery. We focus on small molecule design disrupting IL-6/GP130 D1 domain since a) this is the weakest protein-protein binding interface in the IL-6/IL-6R/GP130 signaling hexameric complex; b) D1 domain is not required for OSM and LIF cytokines, thus achieving design feasibility and reducing side-effects. Through structure-based computational design, combined with synthetic medicinal chemistry and cancer cell biology efforts, we have discovered two major classes of small molecule IL-6 inhibitors: a) MDL-5/MDL-16. We built model of natural compound MDL-A binding to GP130 D1 domain; re-engineered pentendione ring into hydroxybenzene to ease synthetic challenge and added carboxybenzyl ring to gain 10-fold extra binding potency. b) Evista (Raloxifene) and Viviant (Bazedoxifene). We used computational drug repositioning strategy to discover that FDA-approved anti-osteoporosis drugs Evista (Raloxifene) and Viviant (Bazedoxifene) are IL-6/GP130 D1 domain inhibitors. These compounds were proved to molecularly bind to GP130 D1 domain as designed and showed significant effects in IL-6/STAT3 signaling inhibition and breast cancer cellular apoptosis, thus are novel lead molecules to be modified and tested further to become novel anti-metastasis and anti-resistance breast cancer drugs.

15. SUBJECT TERMS
IL-6, GP130, STAT3, breast cancer, drug, MDL-5, MDL-16, Raloxifene, Bazedoxifene

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INTRODUCTION

Interleukin-6 (IL-6) is a key signaling molecule in breast cancer cells. It is responsible for many cellular responses in both cancer and normal cells, including immune response, cell survival, cell death, and proliferation. Unfortunately, IL-6 may also play a key role in the progression of breast cancer from stage I to stage IV cancer (typically associated with a poor prognosis among breast cancer patients). This change to a more serious cancer is associated with significantly increased levels of IL-6, which is believed to affect the subsequent proliferation and metastasis of the tumor cells by initiating a complex series of molecular signal pathways, specifically the IL-6/JAK2/STAT3 pathway. Therefore, we are examining a new strategy to combat breast cancers by disrupting the initiation of the IL-6 signaling using small synthetic molecules using the natural product madindoline A as a starting point for our studies. Madindoline A (MDL-A) is known to interact with the IL-6 receptor on the surface of the cell and prevent this signaling event. Modification of the chemical structure of MDL-A and new design using it as a structural template should provide more potent and selective derivatives which may be useful therapeutic agents for the treatment of breast cancer. Thus, a multidisciplinary team has been assembled with expertise in computational chemistry, synthetic chemistry and cancer biology in order to design and synthesize the new compounds, and in biochemical and cellular assays to assess the effectiveness of these agents. To date, more than twenty novel analogues have been synthesized and partially tested for their ability to bind to gp130 and inhibit STAT3 phosphorylation. The data obtained during the course of our studies into the anticancer properties of these molecules will be utilized to refine and improve upon our model, and our new lead molecules can be further modified and tested with the ultimate goal of developing a useful treatment for drug-resistant and metastatic breast cancers.

BODY

Task 1. Design and synthesize novel madindoline A analogues as inhibitors of the IL-6/GP130 interaction for the treatment of breast cancer.

1. *Computational design and optimization will be carried out throughout the entire project period. (Months 1-24)*

PART ONE

In order to examine the relative binding positions and poses of MDL-A and the novel analogues MDL-5 and MDL-16 (an improved MDL-5 analog for both potency and synthesis), a docking study was carried out. Based on this study, it was determined that both MDL-5 and MDL-16 bind with similar binding conformations as MDL-A (Figure 1D) and do pick up the additional interactions predicted from the extra subpockets in the gp130 D1-domain (see Figure 1C). As a result, the predicted binding energies of the designed analogues were better than MDL-A. MDL-5 and MDL-16 both showed about a -3 kcal/mol improvement in binding energy compared to the MDL-A binding energy. 1.36 kcal/mol difference in binding energies corresponds to 10 fold difference in activity, which is close to the difference in activity observed

experimentally between MDL-5 and MDL-A. It should be noted, however, that there is a statistical error associated with these docking studies of approximately 2 kcal/mol.

The relative stability of the three compounds in the MDL-A binding pocket has also been examined. The binding stabilities of MDL-A, MDL-5 and MDL-16 are represented in atomic fluctuations with respect to their initial binding conformation (Figure 2). Atomic fluctuations were calculated by averaging atomic fluctuations over 20 ns MD simulation. The HFI unit of MDL-A showed instability in the gp130 D1-domain binding pocket, whereas the hydrophobic tail (diketo cyclopentedene ring) of MDL-A showed stability with least atomic fluctuations. The southern tail of MDL-A seems to be very important and keeps it from "flying away" from the binding pocket indicating a more significant interaction. Our computational results are consistent with the previous study which showed that the tail portion of MDL-A is important for its activity and supported the results of Saleh et al.[1], which demonstrated that a compound containing only an HFI unit was not capable of inhibiting gp130 homodimerization. Our MDLs MD simulation studies showed consistent results. MDL-5 and MDL-16 showed very stable confirmation at D1-domain binding pocket (Figure 2). Average root mean square fluctuations (RMSf) for MDL-16, MDL-5 and MDL-A were 1.6 (±0.4), 1.7 (±0.4) and 4.0 (±1.1), respectively. Stable binding dynamics of MDL-5 and MDL-16 validated our design idea and predicted that these compounds should demonstrate improved activity. In order to calculate the absolute binding energy of MDL-A, MDL-5 and MDL-16, we calculated binding free energy of each complex using MMPBSA methods.

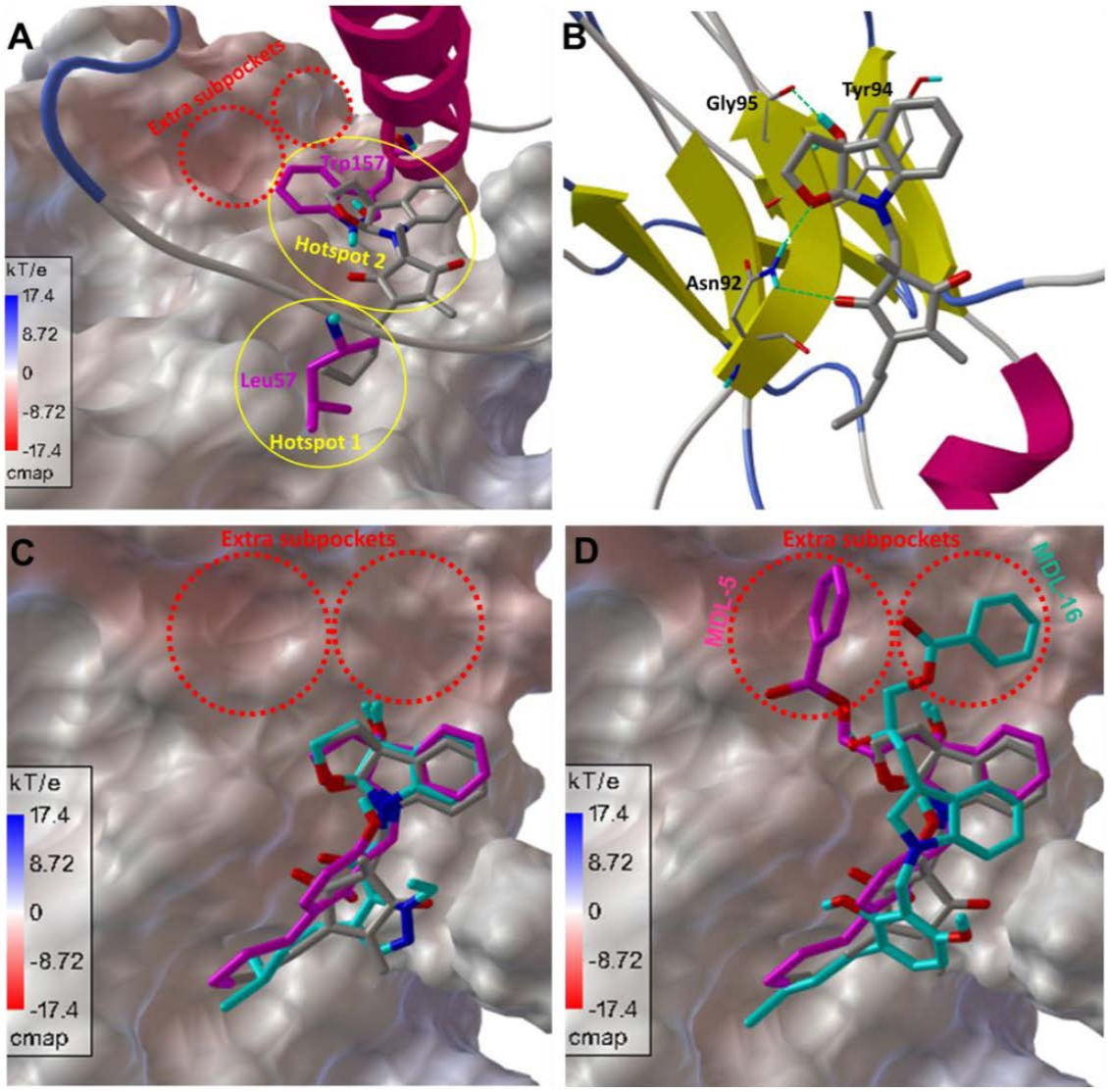


Figure 1. Binding modes of designed MDL-A analogues: A. gp130 D1-domain in electrostatic surface representation; IL-6 in ribbon representation. The two larger yellow ellipses indicate two major binding "hot spots" between IL-6 and gp130. The small dotted red circle points to an empty polar extra subpockets. **B.** D1 domain in ribbon representation and MDL-A in thick ball-and-stick. **C.** Modified southern pentendione ring of MDL-A: Overlaid binding modes of MDL-A (grey color), pyrazole analogue (cyan color) and hydroxyl analogues (magenta color) are shown in ball and stick representation on gp130 D1-domain surface; **D.** Binding modes of MDL-5 (magenta color) and MDL-16 (cyan color) are shown overlaid with MDL-A on gp130 D1-domain. MDL-5 and MDL-16 captures additional binding interactions from extra subpockets shown as small dotted red circles.

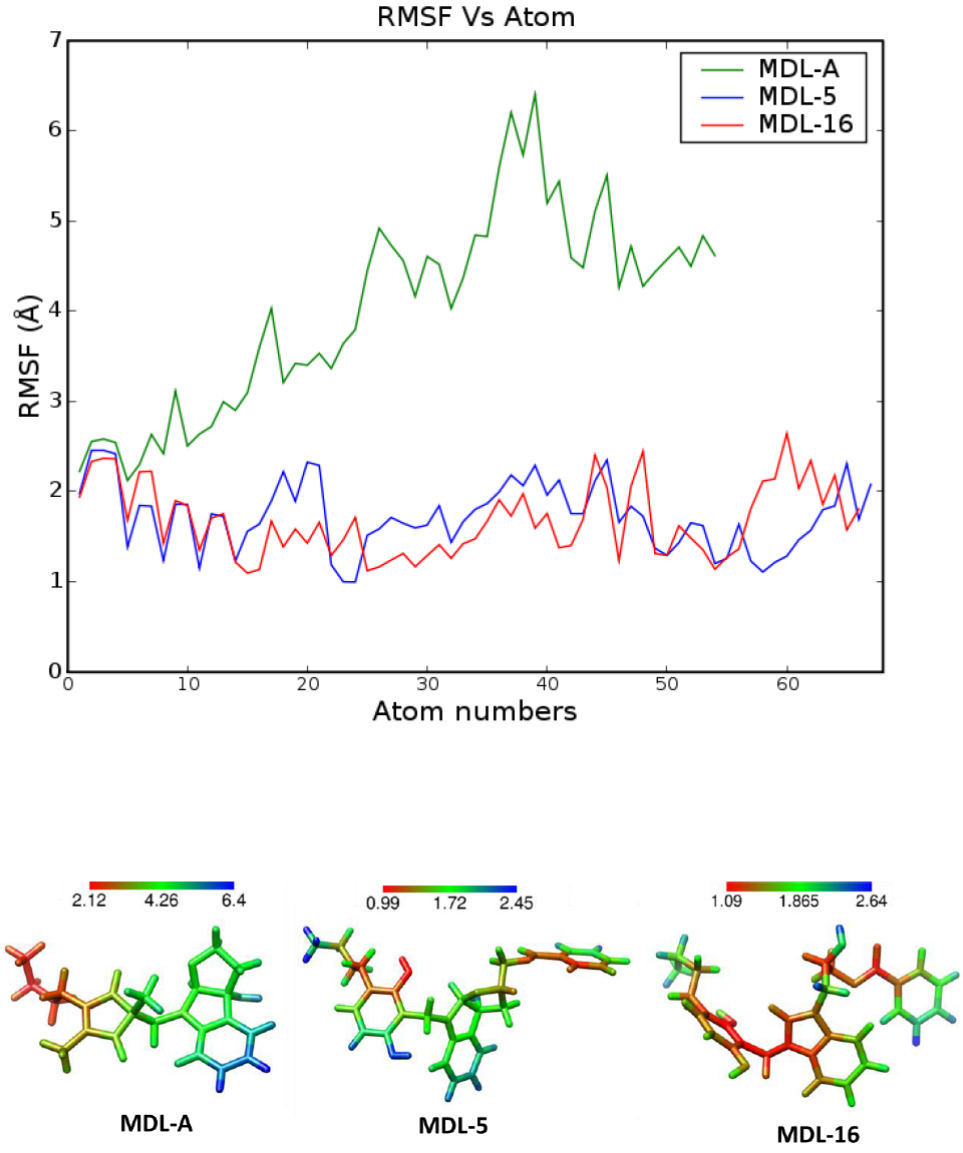


Figure 2. Atomic fluctuations of MDL-A, MDL-5 and MDL-16 during a 20 ns MD simulation. MDL-16 (red color RMSf) and MDL-5 (blue color RMSf) showed stability at the binding pocket compared to MDL-A (green color RMSf). Atoms are colored based upon their RMSf (Root mean square fluctuations).

The results of the stability analysis were confirmed by looking at a per residue free energy calculation. The binding free energy contributions of each amino acid residues of D1-domain were calculated for each complex (IL-6/D1-domain, MDL-A/D1-domain, MDL-5/D1-domain and MDL-16/D1-domain). All complexes showed overlap in amino acid residues which are involved in binding interactions and contribute to the overall bind free energies (Figure 3). For the MDL-A/D1-domain complex, amino acid residues which interact with the tail of MDL-A contribute most towards binding

free energy. For MDL-5 and MDL-16 additional free energy was gained by interactions with the extra subpocket amino acid residues.

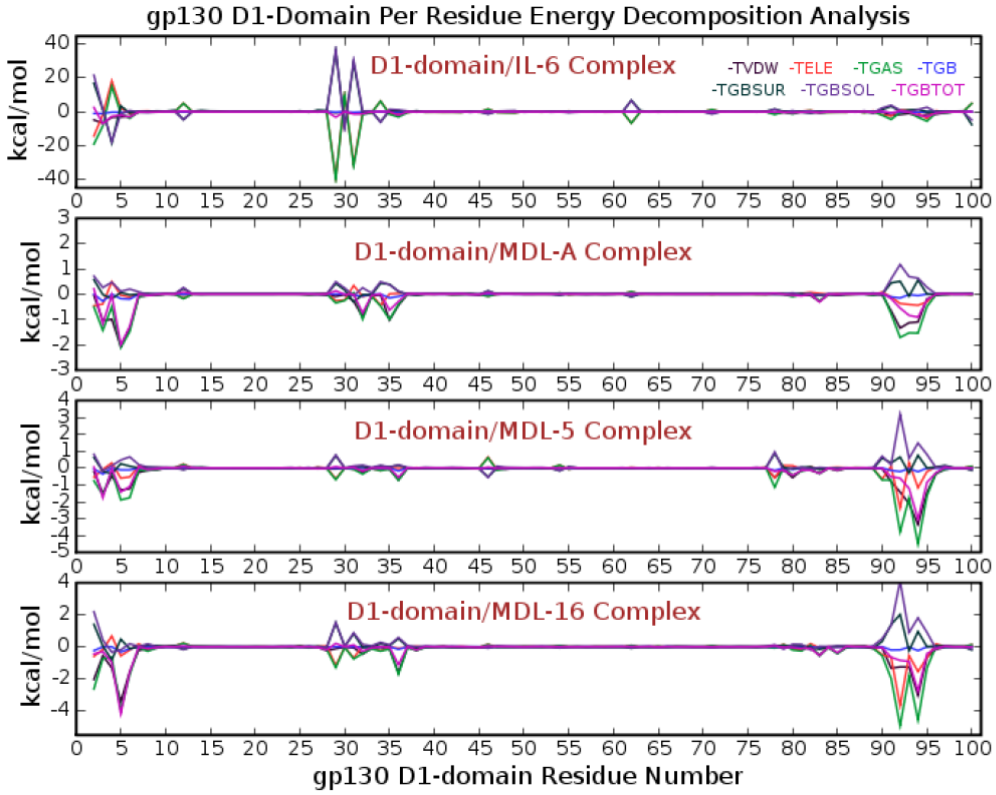


Figure 3. Binding free energy decomposition on per residue contributions for IL-6/D1-domain complex, MDL-A/D1-domain complex, MDL-5/D1-domain complex and MDL-16/D1-domain complex: *EVDW*: van der Waals energy component; *EELE*: Electrostatic energy component; *EGAS*: Gas phase energy component (*EVDW* + *EELE* + *Eint*); GGB: electrostatic solvation energy using GB model (polar contribution); *GGBSUR*: nonelectrostatic solvation components (nonpolar contributions); *GGBSOL*: Solvation free energy component; *GGBTOT*: (*EGAS*+*GGBSOL*).

An analysis of each of the forces responsible for the binding interactions of MDL-A, MDL-5, and MDL-16 has also been carried out by examining the enthalpy *(ΔH)* and entropy *(ΔS)* of the system, the thermodynamic forces responsible for binding free energies $(\Delta G_{binding})$. By utilizing the additive nature of the enthalpy term in the binding free energy calculation, each component was separated into polar, nonpolar, electrostatic and hydrophobic terms. The results (not shown) demonstrated that enthalpy was the driving force for the binding interactions of MDL-16, MDL-5 and MDL-A to the gp130 D1-domain. For all compounds hydrophobic and nonpolar interactions were dominant forces, particularly van der Waals forces. The nonpolar term ($G_{nonpolar}$) of solvation free energy was favorable while the polar term (G_{polar}) was unfavorable for complex formation in all cases. Calculated binding free energies show that MDL-16 is more potent than MDL-5 and which in turn is better than MDL-A.

Milestone 1: we have built reliable small molecule/GP130 D1 binding models at both structural and dynamic/energetic levels for MDL-A, MDL-5 and MDL-16, the most potent small molecule IL-6 inhibitors so far.

PART TWO

In recently years, my lab has developed a novel ligand/protein simulation method called Multiple Ligand Simultaneous Docking (MLSD) [2]. We used it for fragment-based drug design

and drug repositioning/repurposing, a popular drug discovery strategy nowadays (existing drugs for novel targets). We successfully found that anti-inflammatory drug, celecoxib, is a weak inhibitor of STAT3 oncoprotein [3]. In a similar fashion, we have tried it on the D1-domain here.

We built a small library of feature fragments from key interacting residues (Leu57 and Trp157) of IL-6, inhibitor MDL-A and the more potent analogues MDL-5 and MDL-16. The feature fragments are listed in Figure 4. Here the aromatic indole fragment is a key moiety to mimic residue Trp157 of IL-6, and the ButylPhenyl fragment is used to displace the hydrophobic Leu57 of IL-6. Learning from the hot spot binding residues of IL-6 and the feature fragments of inhibitor MDL-A, our strategy is to identify drug scaffolds with stronger affinities. To avoid fragments with undesired drug ADMET properties, drug scaffolds structurally or chemically similar to the feature fragments were identified by sub structure or similarity searches on a drug scaffold database and DrugBank. Figure 5 lists the drug scaffolds identified, which were grouped into 2 pools: aromatic and nonpolar. The aromatic scaffolds in pool 1 favor binding to the Trp157 site, and the nonpolar scaffolds in pool 2 are for the Leu57 site or the extra subpockets. Piperidine and cyclohexane, very common six member rings in drugs, were used to replace the aliphatic tail of MDL-A to improve the binding affinity for the deep hydrophobic Leu57 binding pocket on the gp130 D1 domain.

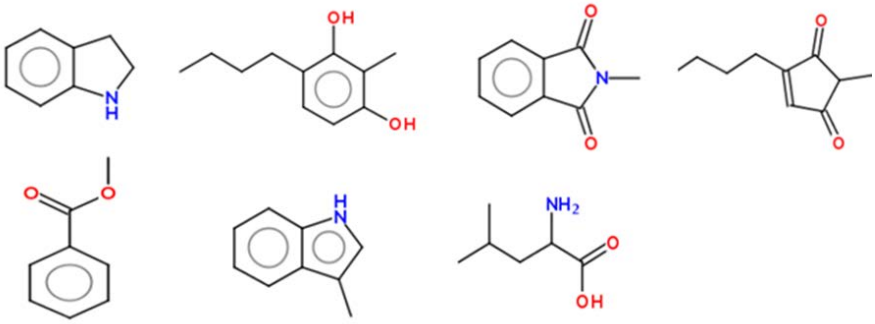


Figure 4. Feature fragments from Trp157 and Leu57 residue of IL-6, inhibitor MDL-A and its analogues to mimic the hot spot residues of IL-6.

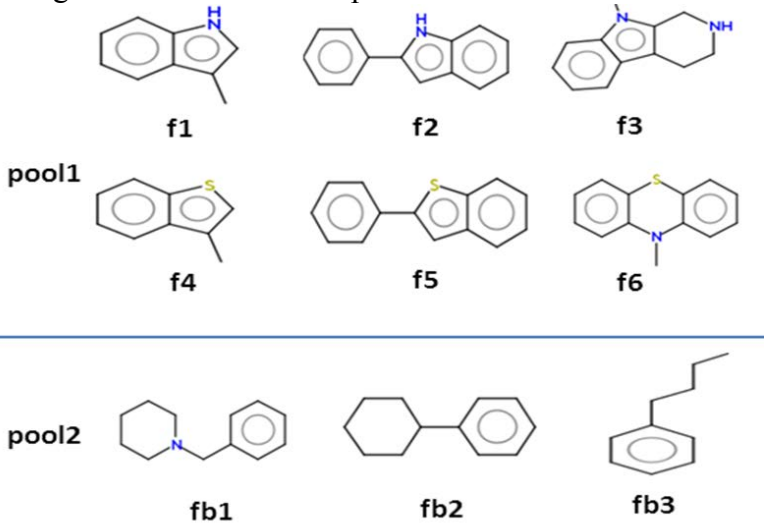


Figure 5. Structure of drug scaffolds identified for the binding hot spots of gp130.

To improve binding affinity, we applied MLSD to dock multiple drug scaffolds in a concerted way to the 2 binding hot spots of GP130, effectively disrupting multiple key residues of IL-6 binding to GP130 D1 domain. Briefly, the combinations of two drug fragments, one from pool 1 and the other from pool 2, were used as inputs for MLSD docking screening. Briefly, we found that f5/fb1 fragment combination is the most optimal. We linked this two fragments to

generate an *in silico* structural template and searched DrugBank, and found that Evista/ Raloxifene and Viviant/Bazedoxifene, two anti-osteoporosis drugs, are also inhibit IL-6/gp130 interaction [4]. Figure 6 shows their binding to D1 domain.

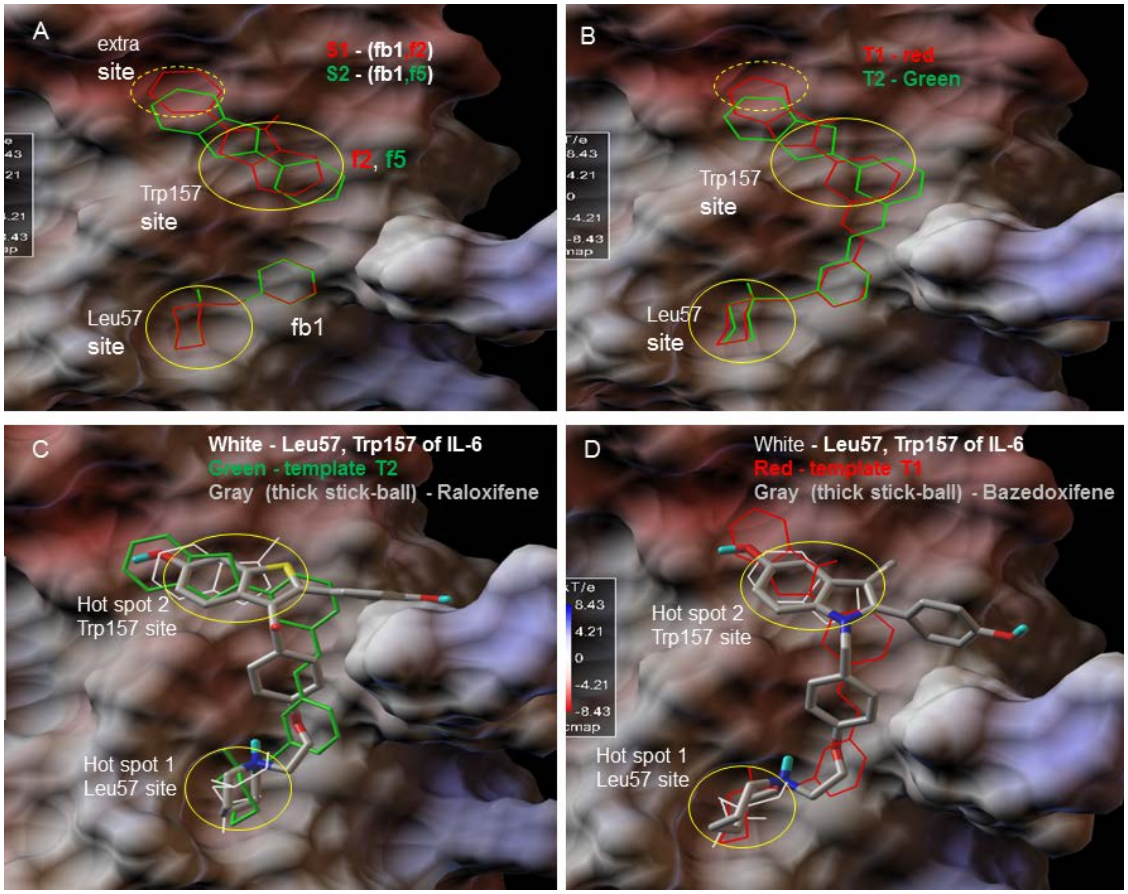


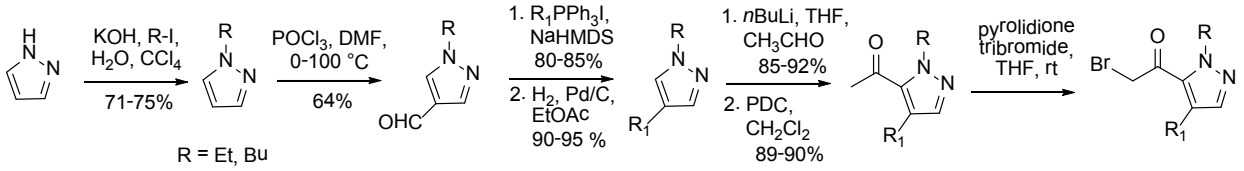
Figure 6. Docking modeling of the best drug scaffolds, the virtual templates, and drug candidates to GP130. A. In top hits of fragment set S1 (green) and S2 (red color), fragment fb1 (phenyl-piperdinyl) occupied the Leu57 hot spot, while f2 (2-Phenylindole) and f5 (2-Phenyl-1-benzothiophen) bound to the Trp157 binding site of GP130 with a binding energy of -9.4 kcal/mol and -9.5 kcal/mol, respectively. B. Tethering fragments in hit S1 and S2 gave virtual template compounds T1 (red) and T2 (green), respectively. Docking free energies and binding modes of template compounds T1 (-9.1 kcal/mol) and T2 (-9.3 kcal/mol) are close to the corresponding fragments in hits S1 and S2. C. Drug Raloxifene was identified as a novel inhibitor of GP130 D1 by similarity searching for T2 in DrugBank. Docking of Raloxifene (thick gray ball-and-stick) to GP130 D1 showed that piperidinyl and 1-benzothiophen moieties mimic the native Leu57 and Trp157 (in white lines) of IL-6 in the hot spots, respectively. D. Docking of drug candidate Bazedoxifene (thick gray ball-and-stick) to GP130 D1 domain. The indole moiety and seven-membered ring azepanyl of Bazedoxifene almost overlaps with the native Trp157 and Leu57 residues (white lines) of IL-6, respectively.

Milestone 2: We have discovered that anti-osteoporosis drugs, Raloxifene and Bazedoxifene, are IL-6 inhibitors.

2. *Initial laboratory synthesis of madindoline A analogues for in vitro biological testing will be carried out. (Months 1-24)*

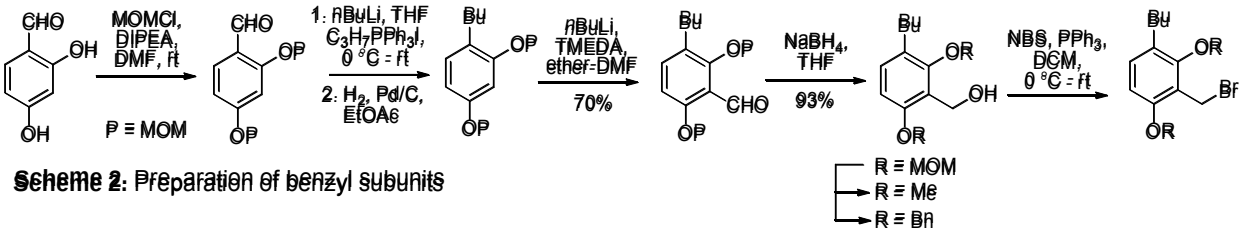
The first major task with regard to validating the hypothesis in the proposal has been to synthesize the desired analogues of madindoline A. Retrosynthetically, the approach to the proposed analogues relied upon disconnection of the molecules into two halves, the hydroxyfuroindoline portion and a "southern" pyrazole or benzyl-containing portion, as indicated in the proposal. An efficient synthesis of the "northern" hydroxyfuroindoline portion of madindoline employing the Sharpless epoxidation and concomitant cyclization of tryptophol has previously been reported by Smith and coworkers [5]. This strategy was employed for most of the analogues synthesized to date and was also anticipated to be useful for the preparation of substituted hydroxyfuroindolines through slight modification of reaction conditions. Since this chemistry has already been "established", we first set our sights on synthesizing the "southern" portions of the proposed molecules.

The pyrazole fragments were synthesized as shown in Scheme 1. Pyrazole was readily alkylated using an alkyl iodide, either iodoethane or iodobutane. The alkylated pyrazoles were then formylated at the C4 position using Vilsmeier-Haack conditions. At this stage, Wittig olefination of the aldehyde and hydrogenation of the resulting olefin was employed to introduce the alkyl chain. The pyrazole could then be functionalized regioselectively at the C5 position upon treatment with *n*-butyllithium and acetaldehyde. The resulting alcohol was subsequently oxidized to the methyl ketone with PDC. Bromination of the ketone could then be accomplished with pyrrolidine tribromide, providing the substrate for alkylation with the hydroxyfuroindoline. Conversely, attempts to directly introduce the brominated acetyl group using bromoacetyl bromide resulted in only poor yields of the desired bromide.



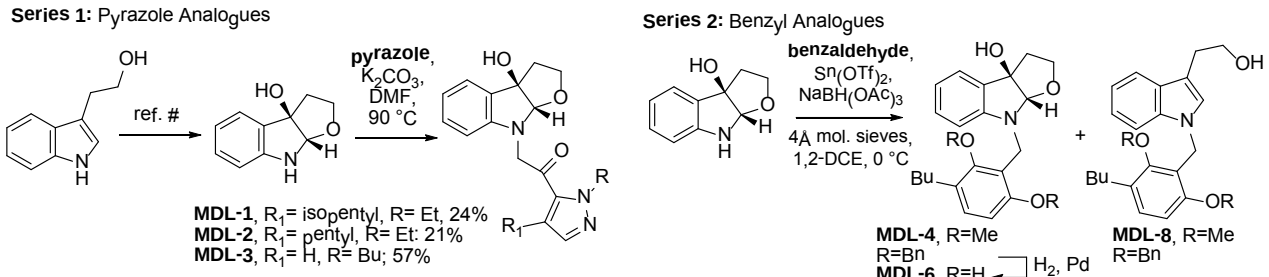
Scheme 1. Preparation of pyrazole subunits

The synthesis of the benzyl fragment required for the second series of analogues is shown in Scheme 2. In this case, the synthesis began with protection of 2,4-dihydroxybenzaldehyde as the methoxymethyl (MOM) ether derivative. Conversion of the aldehyde to the styrene derivative via Wittig olefination and subsequent hydrogenation produced the resorcinol derivative in a manner analogous to the introduction of the pyrazole alkyl chain. This compound could then be formylated by lithiation and trapping of the anion with DMF. This aldehyde would serve as the substrate for a reductive amination reaction with the hydroxyfuroindoline. Alternatively, reduction of this aldehyde to the primary alcohol followed by bromination of the resultant alcohol gave rise to the corresponding bromide which could be employed in an alkylation reaction (again similar to the plan for the pyrazoles, above).



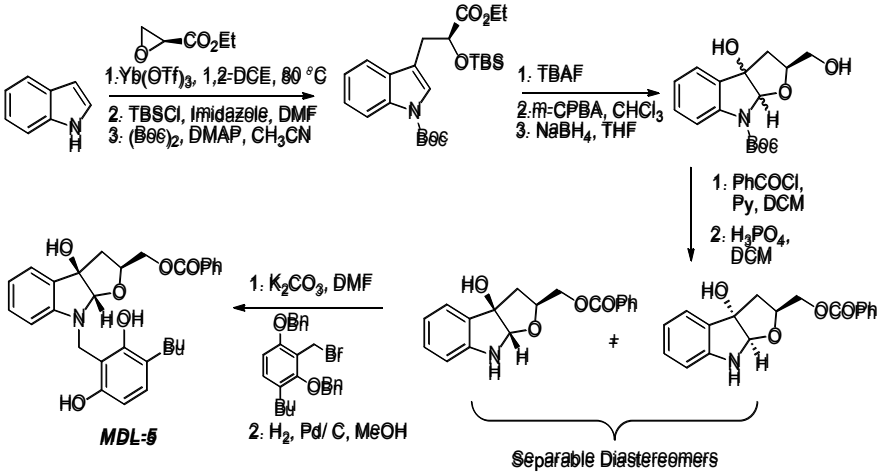
Scheme 2. Preparation of benzyl subunits

The combination of the the HFI unit and the "southern" pyrazole half, was initiated by alkylation of aryl halide in the case of **MDL-1 - MDL-3 (**Scheme 3), albeit in relatively low yield. In the case of the benzyl analogues, this was initially accomplished via reductive amination of the benzaldehyde with the aniline nitrogen of the HFI moiety. Not surprisingly, however, the acidic conditions caused the tricyclic ring system to open up and upon elimination of the hydroxyl group, also provided the corresponding tryptophol derivatives (e.g., **MDL-8,** Scheme 3). Alkylations using the benzylbromides synthesized in Scheme 2 provided slightly better results. The major obstacle in the synthesis of the benzyl analogues, however, has been the late-stage deprotection of the phenols. In an attempt to improve the yields obtained from deprotection of the MOM groups, we have also looked at Me and Bn protecting groups, although a completely satisfactory solution has not yet been found.



Scheme 3. Linking the hydroxyfuroindoline with the pyrazole or benzyl portions.

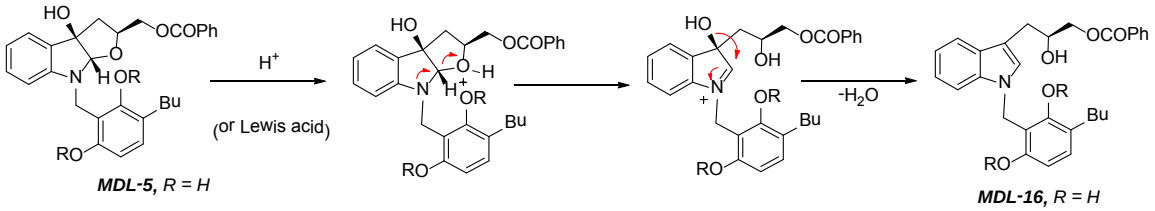
In order to test the hypothesis that adding substituents to the hydroxyfuroindoline ring would improve gp130 binding, we also synthesized MDL-5 (Scheme 4). This was accomplished by using ethyl glycidate to introduce the necessary stereogenic center. In this case, however, Sharpless epoxidation failed to provide the desired product in sufficient yield, presumably due to the decrease in reactivity due to substitution. Therefore, the epoxidation was simply carried out using mCPBA, resulting in the production of 2 diastereomeric products. Upon reduction, acylation, and deprotection of the indole nitrogen, these products were separable by column chromatography. Finally, alkylation and hydrogenation provided MDL-5, albeit in low yield.



Scheme 4. Initial MDL-5 synthesis.

In an effort to understand this low yield, we examined these reactions more closely. Although it appeared that the alkylation reaction proceeded very slowly, only starting material

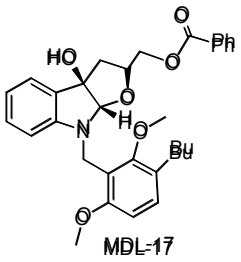
and product were found as major components in the reaction mixture. In the hydrogenation reaction, however, an unexpected byproduct was also isolated suggesting that the hemi-aminal moiety of the HFI unit is somewhat unstable. In this case, it appears to rapidly undergo a ring opening and elimination reaction in the presence of acidic or Lewis acidic reagents to produce the indole (or more appropriately the tryptophol unit) as the core of the northern half of the molecule as shown in Scheme 5. This new compound was assigned the code MDL-16. Interestingly, the tryptophol, which we had previously considered as a possible "bioisostere" of the HFI unit based on similar hydrogen bonding potential, overall size, and geometry, was found to be slightly more active than MDL-5 itself in the gp130 binding assay (see Task 2 below). In addition, the ring opening does not appear to be as significant in systems that lack the additional benzoyl substituent found in MDL-5, suggesting that the HFI unit in MDL-A may be somewhat more stable that of MDL-5. At this stage, however, direct comparison of these ring systems has not been rigorously examined.



***MDL-5**, R = H* ***MDL-16**, R = H*

Scheme 5. Proposed mechanism for the ring opening transformation of the HFI unit to the indole in the presence of acid.

In an effort to prevent the formation of the MDL-16 and improve the yields of MDL-5 or related compounds, a number of alternative protecting group strategies were explored. These included using the MOM (methoxymethyl) groups and Me groups explored during the initial synthesis of the benzyl subunit (Scheme 2, year 1 report). In both of these cases, however, these attempts provided little to no improvement over the hydrogenation as MDL-16 was also formed at the expense of MDL-5 during these deprotection reactions. For example, a variety of conditions attempted for the removal of the MOM groups is included in Table 1. These efforts did, however, provide us with an opportunity to make the MDL-5 analogue containing the dimethoxy substituted benzyl ring (MDL-17, right) in order to look at the effect that substitution of the phenols has on the ability to inhibit STAT3 phosphorylation. This compound is able to be generated in much larger quantities than MDL-5.

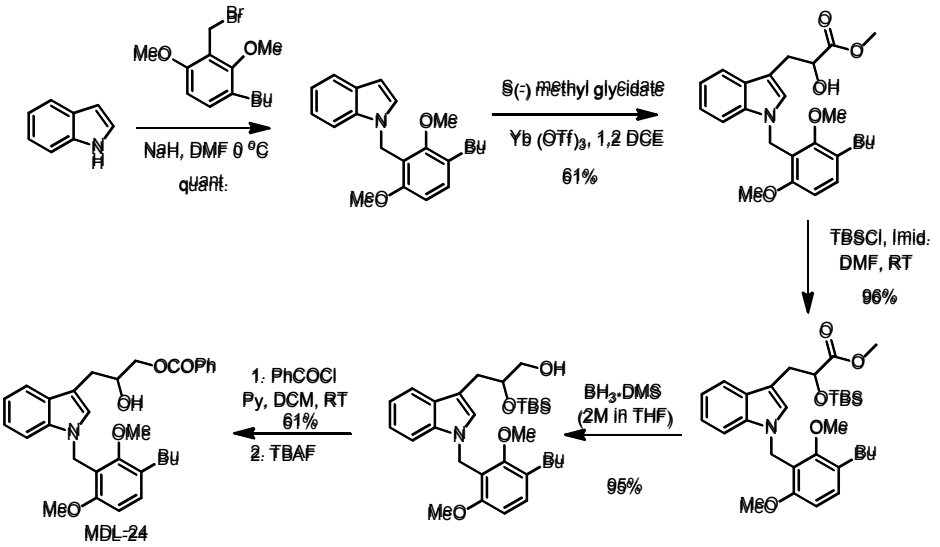


MDL-17

Table 1. Experimental conditions employed in an effort to remove the MOM protecting groups in the synthesis of MDL-5.

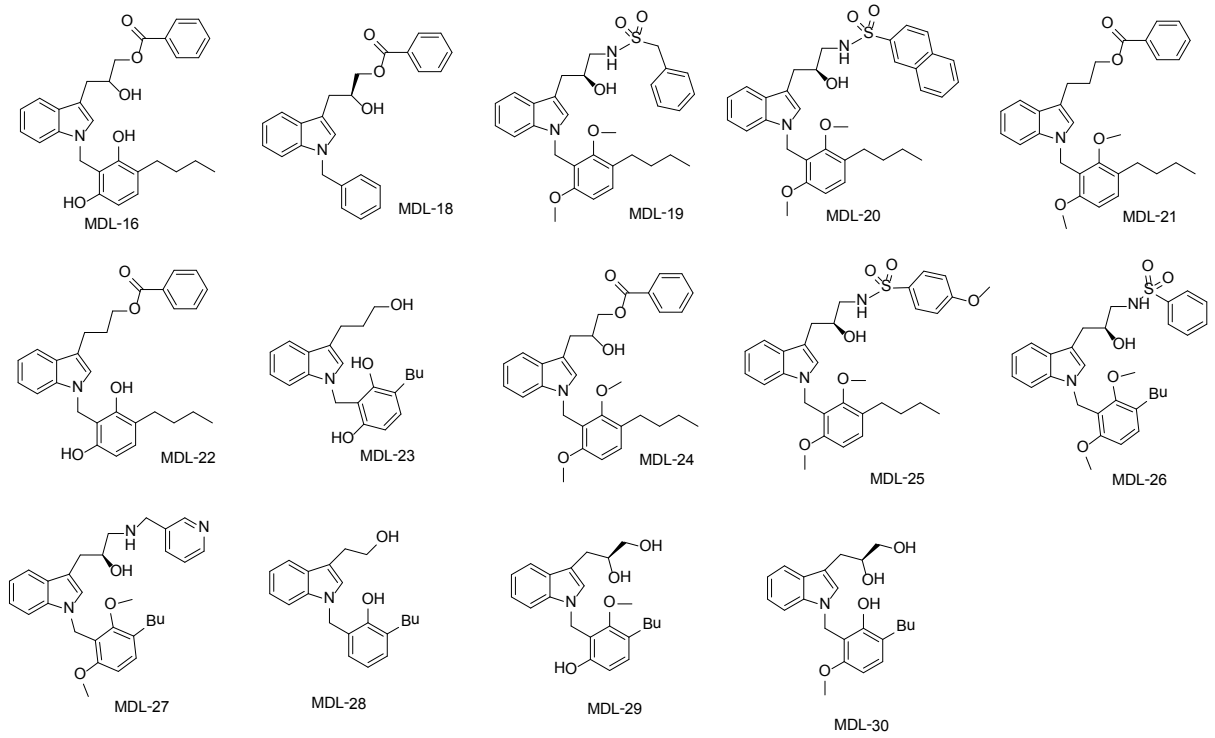
Sl .no	Reaction conditions	Results
1.	10% methanolic HCl	10% yield, multiple spot and base line material
2.	5% methanolic HCl	Deproctected only TBS group; MOMs were intact
3.	Ethylene glycol, 140 °C, 48 h	Very slow.
4.	*n*-PrSH, $ZnBr_2$, RT 5 min	Unknown products
	n-PrSH, $ZnBr_2$, 0 °C, 30 min	10% yield, Mixture of products(only deprotected TBS group + only one MOM group)
5.	*n*-PrSH, $ZnBr_2$, -15 °C, 3 h	15 % yield, Mixture of products(only deprotected TBS group + only one MOM group) and these were difficult to separate
6.	TMSBr, -78 °C to 0 °C 4h	Multiple spots
7.	PPTS, *n*- Butanone	Mostly baseline material
9.	PTSA.H_2O/Toluene	Multiple spots

Unable to effectively prevent the synthesis of MDL-16, we decided to embrace the formation of the product after discovering its potency relative to MDL-5. A thorough computational study was initiated to look at the relative binding conformations and energies of MDL-A, MDL-5, and MDL-16 to gp130 (previously described). Confident that this material could be synthesized more efficiently than MDL-5, we set out to optimize its synthesis and explore the structure-activity relationship of this novel indole class of compounds. Although this deviates slightly from the initial series of compounds proposed in the application, the lead identified through experimentation shows significant promise and should be able to more effectively be modified through synthetic manipulation than MDL-5. It is also expected to demonstrate increased stability. As anticipated, the indole core can rapidly and efficiently be functionalized to provide the methoxy protected MDL-16 derivative MDL-24 as shown in Scheme 6. Surprisingly, low yields are still obtained upon deprotection of the phenolic protecting groups in molecules of this type. This may be due in part to the application of acidic conditions in the presence of the indole and may be resulting in cyclization of an alcohol (or phenol) onto the indolenine generated via protonation of the 3 position of the indole. In this case, we have yet been unable to identify any byproducts of the reaction to confirm or deny this hypothesis. A literature search, however, revealed no similarly substituted benzylic indoles, indicating that reactivity and stability of this type of compound has not yet been reported.



Scheme 6. Synthesis of MDL-24 via modified route.

Regardless, a series of analogues has been synthesized using this approach. In addition to the dimethyl ethers (i.e. MDL-24), these compounds were designed to examine the importance of various functional groups in the MDL-16 molecule, including hydrogen bonding in the benzylic ring (MDL-18, -28, -29, and -30), the role of the hydroxyl substituent on the indole C3 chain (MDL-21 and -22), extension of chain length (MDL-23), the ability to prepare more hydrolytically stable compounds (MDL-19, -20 -25, -26, and -27), and the impact of benzoyl substitution. Attempts to synthesize compounds containing an ether linkage to the aryl ring on the C3 chain have not yet been successful.



Scheme 7. MDL-16 and structurally similar analogues.

Finally**,** attempts to synthesize compounds containing a hydroxymethyl substituent at the C5 position of the indole in order to capture additional interactions with pockets on the benzene

ring side of the indole have failed to provide the originally proposed products. In large part, this is due to the fact that a hydroxymethyl substituent at this position of an indole is prone to an elimination reaction due to the donation of the nitrogen lone pair. In our case, this intermediate appeared to be generated under a number of reaction conditions and was specifically observed during reactions with reducing agents which returned only the C5-methyl derivative derived from delivery of a hydride to the reactive intermediate with loss of the hydroxyl group. This hydrogen bond donor could still be installed; however, in this case it will require the addition of a second carbon atom between the alcohol and the indole ring.

Milestone 3: MDL-5 (ring closure) and MDL-16 (ring opening) IL-6 lead inhibitors were discovered; and further analog synthetic strategy was mapped out.

Task 2. In vitro and in vivo studies of the proposed inhibitors.

PART ONE

MDL-A has been reported to show direct binding at the extracellular domain of gp130 by Saleh and coworkers [1]. We decided to use the same type of direct binding assay as reported in this paper to assess the binding of our analogues. Therefore, we purified recombinant gp130 protein (gp130-Fc-HA) by expressing in HEK293Tcells and purified by protein A affinity chromatography from the medium of transfected cells. Immunoblotting with an anti-HA antibody confirmed that the major species corresponded to the predicted gp130 protein (Figure. 7 B).

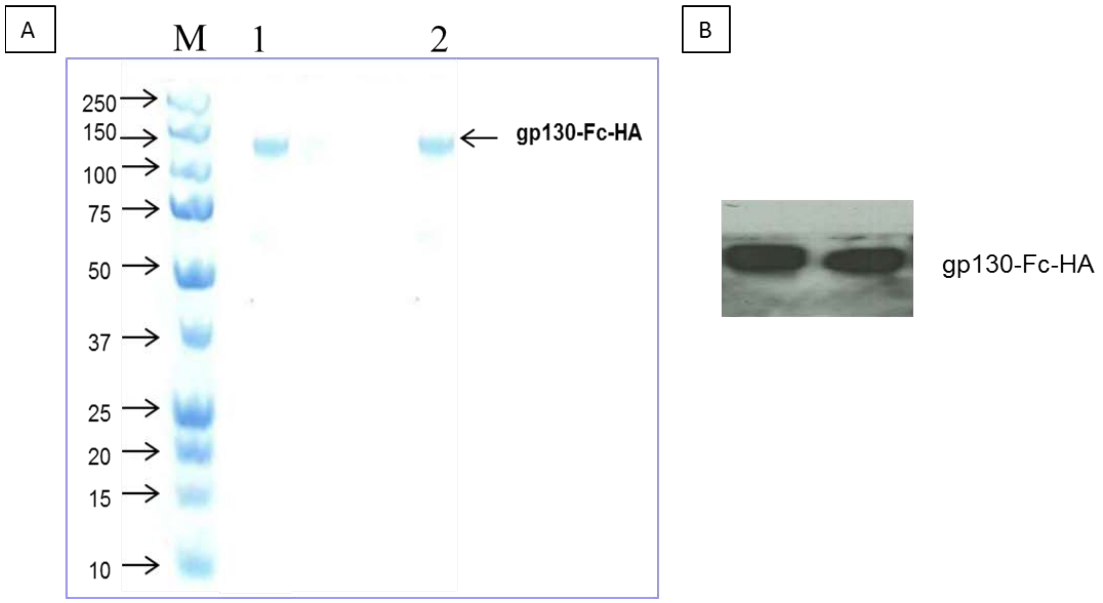


Figure 7: Purification of gp130 extracellular domain: A. Coomassie Blue-stained gel of purified gp130 (amino residue18-615): lane M, molecular weight standard (precision plus protein standards, BIO-RAD); lane 1 and 2: purified gp130-Fc-HA protein. **B.** Anti-HA immunoblot of the purified gp130-Fc-HA protein.

To examine the direct binding and calculate the equilibrium dissociation constant (K_D) of MDL-A analogues with gp130 extracellular domain, surface plasmon resonance analysis was done. Surface plasmon resonance (SPR) analysis was performed using a BIAcore T100. The protein was covalently cross linked to the dextran matrix of the biosensor chip CM5. Successively, various concentrations of MDL-A and MDL-A analogues were injected into the flow cells containing bound protein and no protein. Interactions were monitored in real time and K_D values were calculated by reference-substrate sensogram (figure 8). K_D values (Table 2) were calculated using binding affinity analysis program in Biacore evaluation software version 2.0.

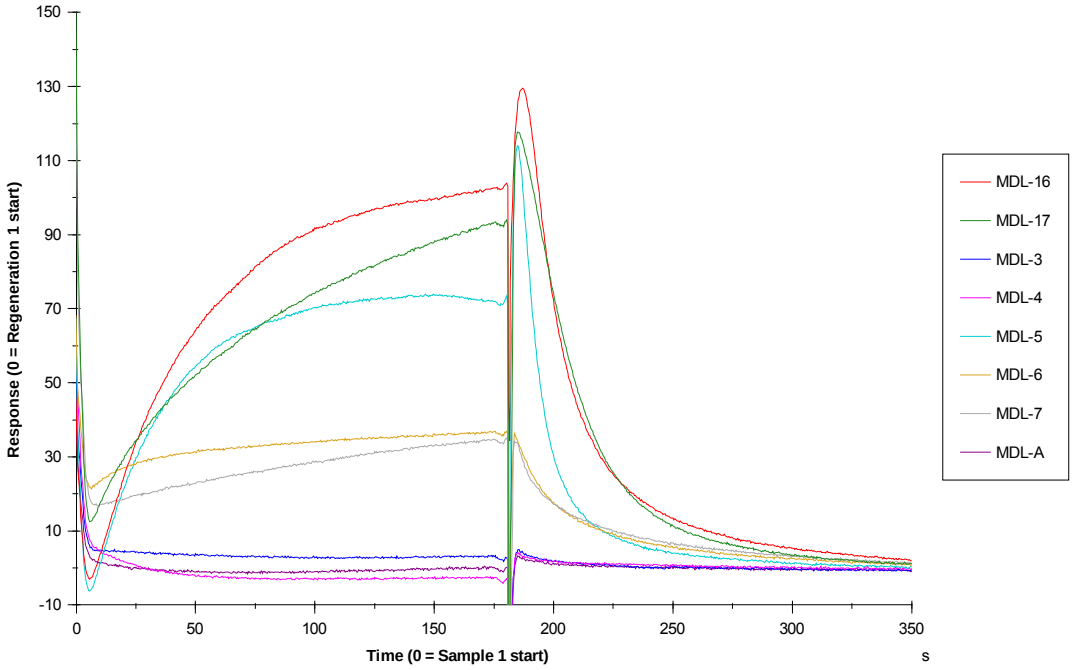


Figure 8. Biacore binding of MDL analogs to D1 domain of GP130.

Table 2. K_D and Binding energy values of MDL-A and MDL-A analogues

MDL-Analogues	AutoDock's Binding Energy (Kcal/mol)	K_D(µM)
MDL-A	-6.0	288
MDL-4	-7.2	N/A
MDL-5	-9.0	37
MDL-6	-6.5	50

MDL-7	-7.5	41
MDL-8	-6.0	41
MDL-16	-9.2	29
MDL-17	-9.0	32

MDL-A analogues also inhibit Stat3 phosphorylation induced by IL-6 (figure 9). Furthermore, the analogues are significantly more potent than MDL-A in terms of inhibition of Stat3 phosphorylation. At 20 μM, MDL-5 and MDL-16 (MDL-5 with furo-ring open) appear to completely inhibit Stat3 phosphorylation induced by IL-6 (25 ng/ml, 30 min) in the MCF-7 cells and the level of pSTAT3 observed is at least somewhat correlated with the K_D values. The lead compounds, MDL-5/-16, show STAT3 selectivity over STAT1 (a tumor suppressor), inhibit STAT3 nulear translation (figure 10 and 11).

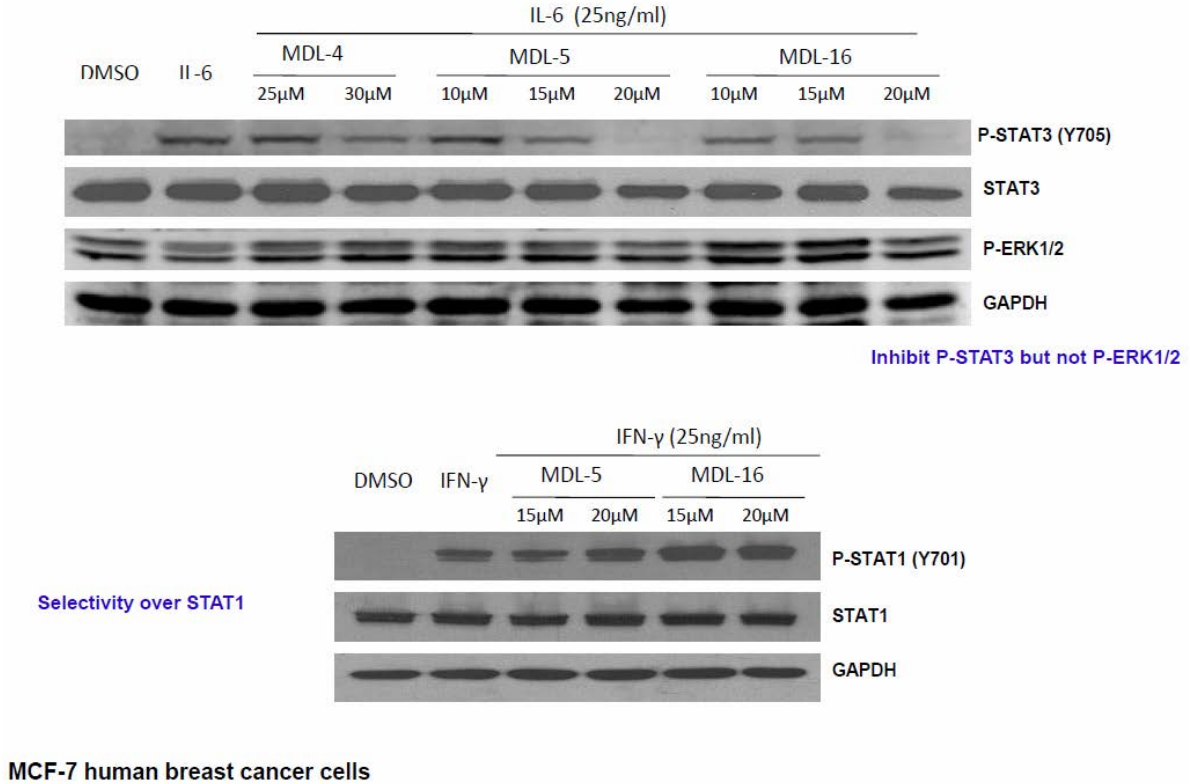


Figure 9. Inhibition of Stat3 phosphorylation by Madindoline A (MDL-A) analogs, but not STAT1.

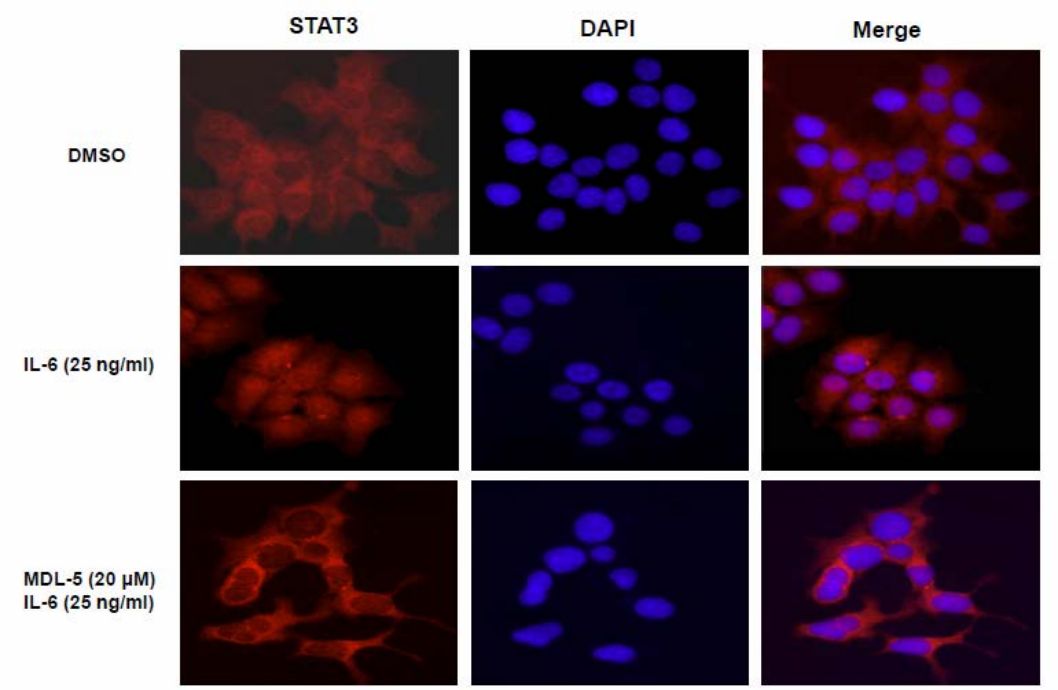


Figure 10. MDL-5 inhibits STAT3 nuclear translocation mediated by IL-6 in MCF-7 cells.

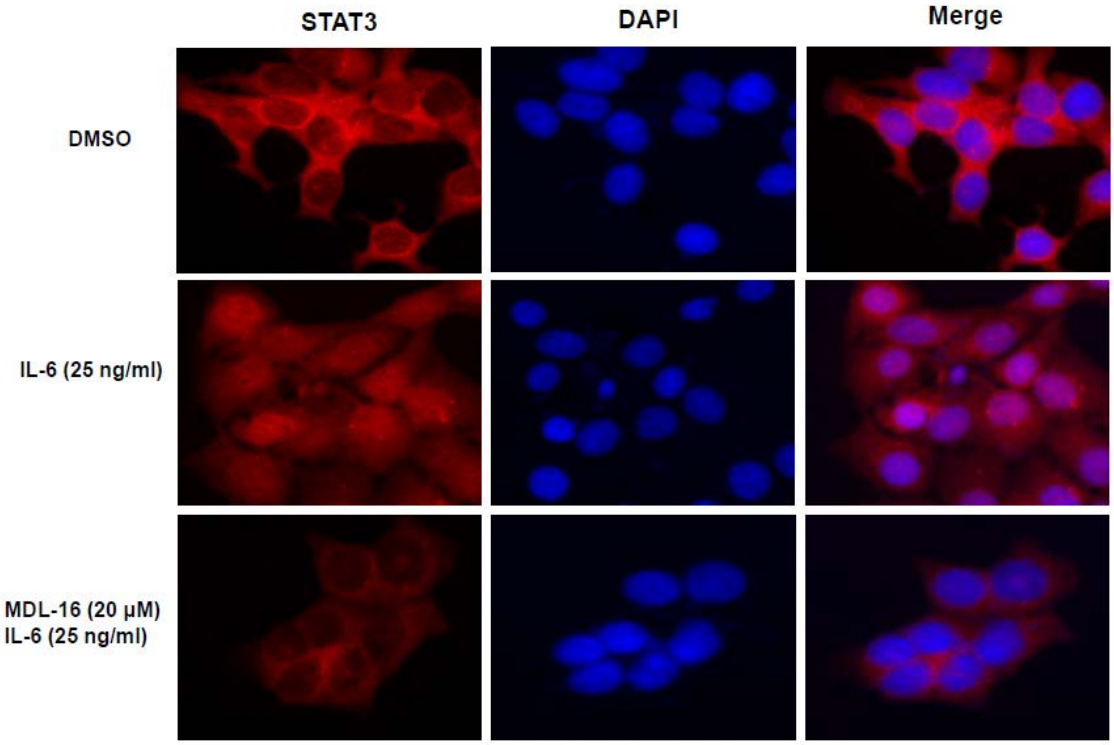


Figure 11. MDL-16 stops STAT3 nuclear translocation upon MCF-7 IL-6 stimulation.

Figure 12 shows MDL-16 selectivity as it inhibits IL-6 but not LIF induced STAT3 phosphorylation, using MCF-7 cell line for testing.

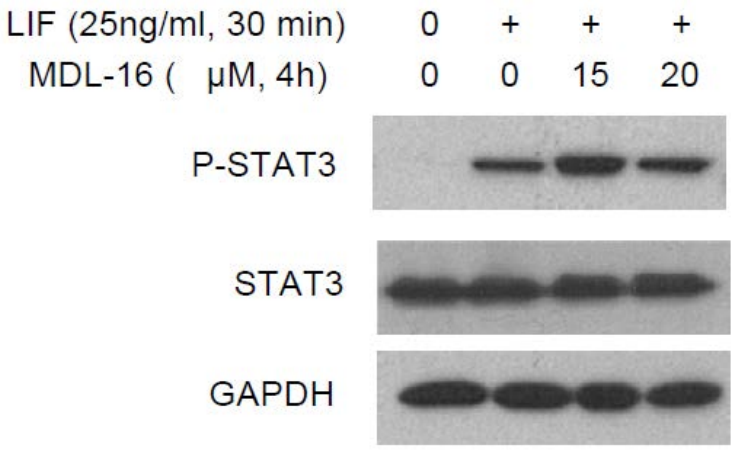


Figure 12. MDL-16 does not inhibit LIF induced STAT3 activation.

Milestone 4: MDL-5 and MDL-16 potency and selectivity were confirmed at both biochemical and cellular levels.

PART TWO

For Raloxifene and Bazedoxifene, we used DARTS assay [6] to confirm their binding to GP130 D1 domain and both efficacy and selectivity were tested at cellular level, comparable to MDL-5 and MDL-16. Since the results were published [4] and attached, it won't be described here repeatedly. In addition, we demonstrated Raloxifene inhibition of STAT3 transcriptional function (figure 13).

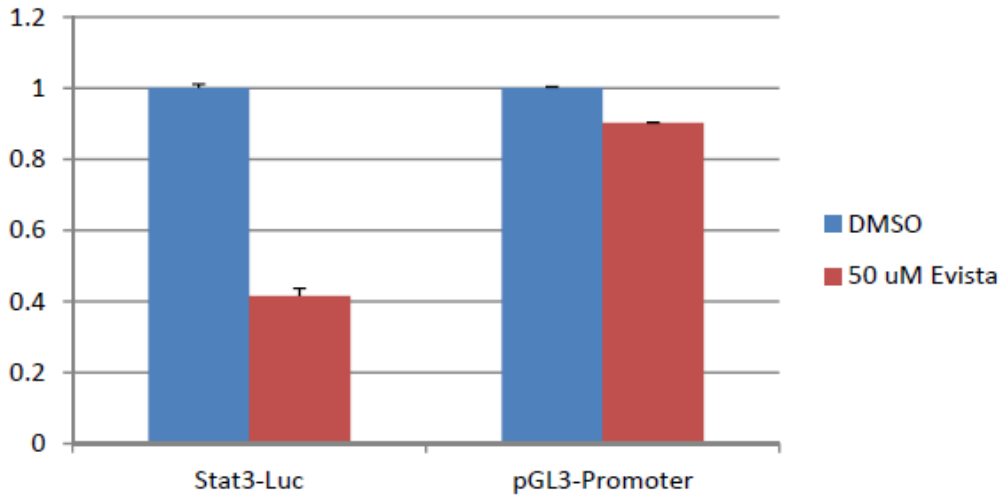


Figure 13. Evista inhibits STAT3 transcriptional luciferase activity, using Hela cell testing.

Milestone 5: FDA-approved anti-osteoporosis drugs, Raloxifene and Bazedoxifene, were confirmed at both biochemical and cellular levels.

KEY RESEARCH ACCOMPLISHMENTS

- Hydrophobic and aromatic interactions are the key design pharmacophores identified through computational modeling and validated through fragment selections.
- Promising lead compounds, MDL-5 and MDL-16, were identified and synthetic routes were established for analog series.
- Two FDA-approved anti-osteoporosis drugs, Evista (Raloxifene) and Viviant (Bazedoxifene), were re-positioned/re-purposed as IL-6/GP130 inhibitors through combined computational and biological investigations.
- Biochemical and biological drug assays were established.
- The four lead compounds showed selective oncogenic IL-6 signaling inhibition and ensuing breast cancer apoptosis, including metastatic triple negative breast cancer cells.
- The research program is ready to move to pro-clinic translational stage in terms of drug development.

REPORTABLE OUTCOMES

1. **Publications and abstracts.**
    - Raloxifene and Bazedoxifene discovery was published at the Journal of Medicinal Chemistry in 2014 (attached).
    - MDL-5 and MDL-16 manuscript had been prepared for the Journal of Medicinal Chemistry, but is under re-writing for the Proceedings of the National Academy of Sciences.
    - An oral presentation was given by the PI on MDL-5/-16 results in the Era of Hope conference in Orlando in August, 2011.
    - A poster presentation was given by a co-I in the American Chemical Society in Denver in August, 2011.
    - A poster presentation was given by a postdoc in the American Chemical Society in San Diego in March, 2012.
    - Ohio Supercomputer Center new release on the project in 2011. https://www.osc.edu/press/biophysicist_targeting_il_6_to_halt_breast_prostate_cancer
    - OSU College of Pharmacy new release on drug repositioning in 2014. http://www.pharmacy.ohio-state.edu/news/college-pharmacy-study-proposes-novel-uses-osteoporosis-drugs-cancer-treatment
    - *Nature Science Business eXchange* reported the Raloxifene and Bazedoxifene as IL-6 inhibitor discovery. http://www.nature.com/scibx/journal/v7/n6/full/scibx.2014.167.html

2. **Research training opportunities.** Two graduate students and three postdoctoral researchers have assisted in these studies. They have been responsible for the computational, synthetic, and biological data obtained for this report. In addition, one of

the graduate students trained obtained her Ph.D. and joined the National Cancer Institute as a research associate.

CONCLUSIONS

The research program has been executed with great success. Four lead molecules are identified with great potential and ready to move forward to pre-clinic evaluations. IL-6 small molecule drug discovery platform is established in the PI's lab. Five graduate students and postdoctoral researchers are trained through the process.

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APPENDICES

(following pages)

Journal of Medicinal Chemistry

Article

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Drug Design Targeting Protein–Protein Interactions (PPIs) Using Multiple Ligand Simultaneous Docking (MLSD) and Drug Repositioning: Discovery of Raloxifene and Bazedoxifene as Novel Inhibitors of IL-6/GP130 Interface

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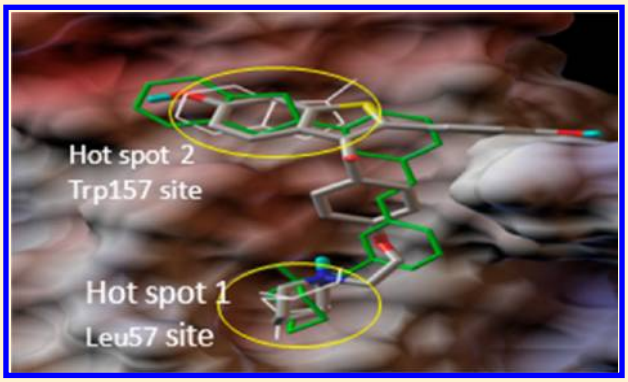
ABSTRACT: The IL-6/GP130/STAT3 pathway is critical for the progression of multiple types of cancers. We report here the discovery of raloxifene and bazedoxifene as novel inhibitors of IL-6/GP130 protein–protein interactions (PPIs) using multiple ligand simultaneous docking (MLSD) and drug repositioning approaches. Multiple drug scaffolds were simultaneously docked into hot spots of GP130 D1 domain by MLSD to compete with the key interacting residues of IL-6, followed by tethering to generate virtual hit compounds. Similarity searches of virtual hits on drug databases identified raloxifene and bazedoxifene as potential inhibitors of IL-6/GP130 interaction. In cancer cell assays both compounds bind to GP130 and demonstrated selective inhibition of IL-6 induced STAT3 phosphorylation and were significantly more potent than the previously reported natural product inhibitor MDL-A. The identified drugs represent a new class of lead compounds with piperidine, benzothiophene, and indole scaffolds to inhibit IL-6 induced homodimerization of GP130. Besides potential direct usage for clinic trials, the two compounds can also serve as lead compounds for optimization to speed the development of drugs selectively targeting the IL-6/GP130/STAT3 cancer signaling pathway.



INTRODUCTION

Interleukin-6 (IL-6) is a cytokine involved in various inflammatory and immune responses, cellular apoptosis, and proliferation, etc.[1] IL-6 binds to IL-6Rα to form a binary complex and then recruits GP130 to form the IL-6/IL-6Rα/GP130 heterotrimer. Furthermore, homodimerization of the IL-6/IL-6Rα/GP130 heteotrimers occurs by interactions between IL-6 of one trimer and the D1 domain of GP130 of the other trimer, forming a hexamer (Figure 1).[2,3] The reciprocal homodimerization of the IL-6/IL-6Rα/GP130 trimers triggers a signaling cascade of phosphorylation of Janus kinases (JAKs) and a downstream effector STAT3, followed by reciprocal dimerization of the Tyr705-phosphorylated STAT3, resulting in STAT3 nucleus translocation, DNA binding, and multiple oncogene transcriptions.[4,5]

Previous studies indicated that IL-6 and the major downstream effector STAT3 are protumorigenic agents in many cancers,[6] supporting both as attractive therapeutic targets. Madindoline A (MDL-A), a natural product compound, was reported as a highly selective, non-peptide antagonist to GP130.[7] It was confirmed that MDL-A binds to the extracellular domain of GP130 and inhibits IL-6 dependent STAT3 tyrosine phosphorylation in HepS2 (hepatocellular carcinoma) cells.[8] However, this natural product cannot be developed into an effective drug because (a) it is no longer available from natural resources, (b) the total synthesis involves many steps, and the low yield makes it not economically practical, and (c) its binding to GP130 is relatively weak (K_d of 288 μM).[8] Although a few MDL-A analogues were identified to inhibit the homodimerization of GP130 via virtual screening and structure based drug design,[9] synthesis of these analogues requires many steps, resulting in yields too low to be practical for drug development. Therefore, it is highly desirable to design and identify novel, small molecule drugs to disable this

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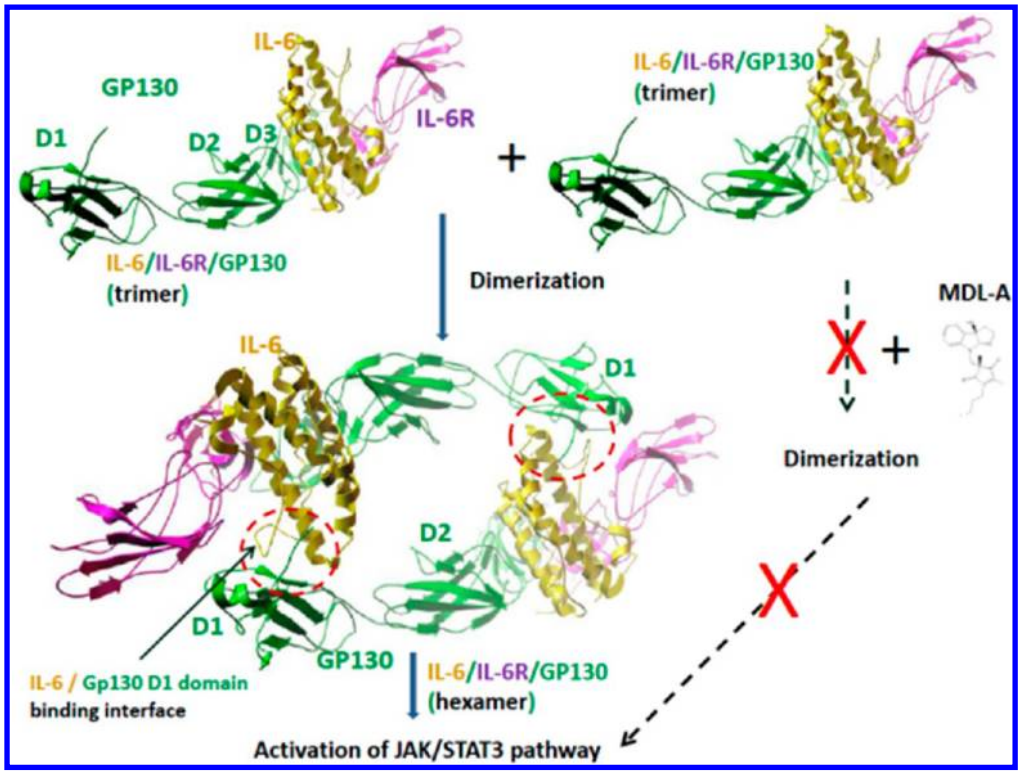

Figure 1. IL-6/GP130/STAT3 signal transduction. IL-6 first binds to IL-6Rα to form the binary complex through site I interactions. The IL-6/IL-6Rα complex then binds to D2 and D3 domains of GP130 through site II interactions and forms trimeric complex IL-6/IL-6Rα/GP130. The subsequent site III reciprocal interaction of IL-6 of one trimer with the D1 domain of GP130 of the other trimer leads to homodimerization of the heterotrimer into heterohexamer complex (PDB code 1P9M).[3] MDL-A binds to the GP130 D1 domain and inhibits hexameric complex formation by disrupting interactions between IL-6 and the GP130 D1 domain.

dimerization of the IL-6/IL-6R/GP130 heterotrimers, offering new options for anticancer therapy.

Small molecule inhibitor design targeting protein−protein interactions (PPIs) is an emerging and challenging area.[9−12] We previously developed a novel computational strategy for fragment-based drug design and discovery by combining MLSD and drug repositioning.[13,14] Our method could be applied to effectively design and identify potential small molecule drugs to disrupt the more challenging IL-6/GP130 protein−protein interface. In the IL-6/GP130 hexameric complex, IL-6, serving as a ligand, binds to the GP130 D1 domain through a few hot spot residues. With the hot spot residues of IL-6 envisioned as multiple fragments, the MLSD method could nicely fit to simulate the multiple residues of IL-6 binding to GP130. The workflow of our approach proceeds as follows:[14]

(1) Focused libraries of drug scaffolds are simultaneously docked into the binding hot spots of target protein (GP130 D1 domain) interface via MLSD.
(2) The multiple fragments bound can simultaneously displace or mimic multiple hot spot residues in protein−protein (IL-6/GP130) interface. Virtual template compounds can then be generated by linking the multiple fragments docked.
(3) Structural or chemical similarity search of the docked fragments and virtual template compounds on drug databases can potentially reposition existing drugs as novel, off-target inhibitors of the new target GP130.

Herein we report the use of our approach to aid the rapid identification of novel inhibitors of the IL-6/GP130 PPI target. We demonstrate the efficiency and efficacy of our approach through the discovery of safe drug compounds with the potential to disrupt the dimerization of GP130. The identified compounds are raloxifene and bazedoxifene. These drugs primarily target the human estrogen receptor (ER), which involve estrogenic actions on bone but antiestrogenic actions on uterus and breast.[15,16] They are commonly used in the prevention of osteoporosis. Our computational prediction has been validated by assays of pancreatic cancer cell lines that express GP130 but not ER. Cell line assays confirmed that raloxifene and bazedoxifene selectively inhibit IL-6 induced STAT3 phosphorylation in the GP130/JAK/STAT3 signaling pathway. IC_{50} values of raloxifene and bazedoxifene in pancreatic cancer (MIA-PACA-2) cell viability assays were 26.3 and 9.7 μM, respectively. Both raloxifene and bazedoxifene demonstrated significantly more potency than the known inhibitor MDL-A (IC_{50} > 250 μM). Since raloxifene and bazedoxifene are approved drugs that have excellent safety profiles and few side effects, they show great potential as drug leads for the development of the much needed, new cancer therapeutics relevant to the IL-6 mediated GP130/JAK/STAT3 signal pathway.

RESULTS AND DISCUSSION

Analysis of Binding "Hot Spots" at Interface between IL-6 and D1 Domain of GP130. Examining the interface between IL-6 and the D1 domain of GP130 in the crystal structure of the hexameric complex (PDB code 1P9M) shows that residues (Leu57, Ala58, Glu59, Asn60, Leu62, Trp157, and Leu158) of IL-6 interact with the D1 domain of GP130. The dimerization of GP130 is mainly hydrophobic as supported by a large increase of entropy from isothermal titration calorimetric

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(ITC) measurements.[3] Previous mutagenesis studies showed Trp157 of IL-6 as the critical aromatic residue at the IL-6/GP130 D1 domain interface.[17] Additional molecular dynamics (MD) simulation using the crystal structure of IL-6 and D1 domain of GP130 complex revealed that Leu57 and Trp157 of IL-6 are the most important "hot spot" residues of IL-6, which contribute most toward the binding free energy for IL-6 and the D1 domain of GP130 interactions.[9] Leu57 is mainly involved in hydrophobic interactions with D1-domain residues. Trp157 shows both polar and nonpolar interactions with the D1-domain residues of GP130. These results helped to define the hydrophobic Leu57 binding site and aromatic Trp157 binding site as the two binding "hot spots" at the interface of IL-6 and the GP130 D1 domain (Figure 2A). In addition, extra subpocket near Trp157 binding site can be used to design more potent and specific inhibitors.

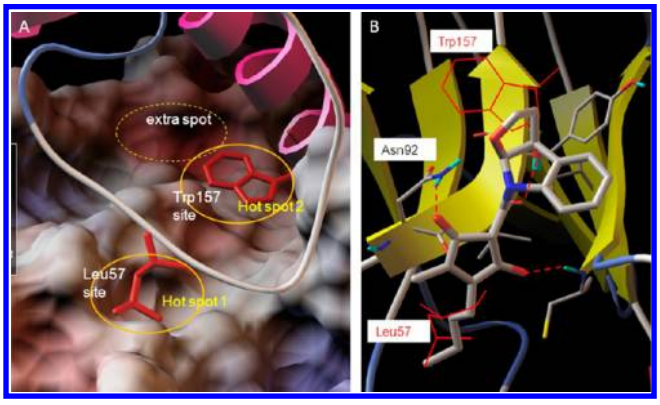

Figure 2. Modeling of binding hot spots at the IL-6/GP130 D1 domain interface. (A) D1 domain is represented as electrostatic potential surface (red, negatively charged; blue, positively charged; white, hydrophobic). IL-6 is in ribbon representation. The two larger yellow eclipses indicate the two main binding "hot spots", Leu57 binding site and Trp157 binding site, between IL-6 and GP130. (B) Binding modeling of MDL-A to the GP130 D1 domain. D1 domain is in ribbon representation, and MDL-A is in thick ball-and-stick rendering. Hydrogen bonds are shown as red dotted lines. MDL-A disrupts both binding spots of the GP130 D1 domain. MDL-A forms three hydrogen bonds with Asn92, Cys6, and the carbonyl backbone of Val93 residues of GP130. The modeling indicates that the long butyl tail of MDL-A displaces Leu57 (thin red line), and the indoline moiety partially disrupts Trp157 (thin red line) of the helix D of IL-6.

To characterize the binding sites and binding modes of the known inhibitor MDL-A on the GP130 surface, MDL-A was globally docked to the GP130 extracellular D1, D2, and D3 modular domains using AutoDock4. Interestingly, MDL-A only binds to the D1 domain but not the D2 and D3 domains, thus suggesting that the compound disrupts only GP130 dimerization. Further analysis of the binding modes of MDL-A indicated that both binding hot spots between IL-6 and GP130 are disrupted by MDL-A (Figure 2B). On hot spot 1, the hydrophobic Leu57 of IL-6 is displaced by the MDL-A aliphatic butyl tail. On hot spot 2, the indoline moiety of MDL-A mimics the Trp157 indole side chain of IL-6.

Identifying Privileged Drug Scaffolds for the Binding Hot Spots of D1 Domain of GP130. We built a small library of feature fragments from the key interacting residues (Leu57 and Trp157) of IL-6, inhibitor MDL-A, and its analogues.[7−9] The feature fragments are listed in Figure 3A. To avoid fragments with undesired drug ADMET properties, drug

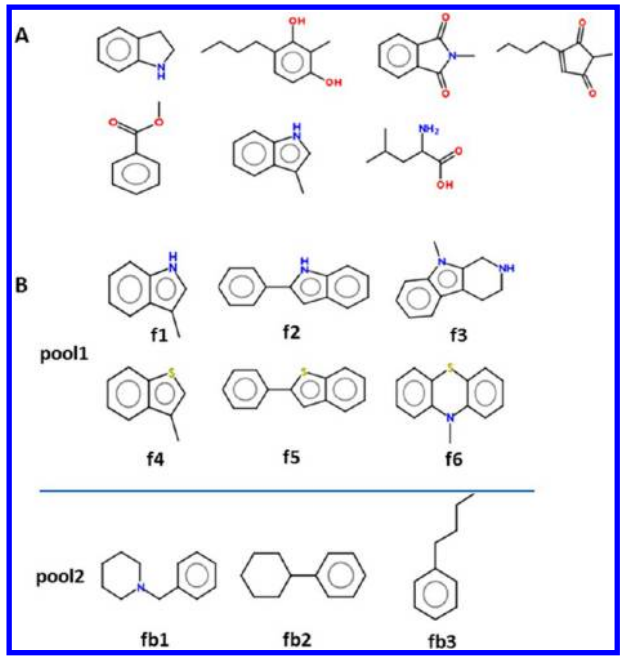

Figure 3. (A) Feature fragments from Trp157 and Leu57 residues of IL-6, inhibitor MDL-A, and its analogues to mimic the hot spot residues of IL-6. (B) Structures of drug scaffolds identified for the binding hot spots of GP130.

scaffolds structurally or chemically similar to the feature fragments were identified by substructure or similarity searches on a drug scaffold database and DrugBank.[18,19] Figure 3B lists the drug scaffolds identified, which were grouped into two pools: aromatic and nonpolar. The aromatic scaffolds in pool 1 may favor binding to the Trp157 site, and the nonpolar scaffolds in pool 2 are for the Leu57 site or the extra subpocket. The hydrophobic piperidine and cyclohexane, very common six-member rings in drugs, were selected to potentially improve the binding affinity for the Leu57 binding pocket on the GP130 D1 domain.

Simultaneous Docking of Two Fragments to Binding Hot Spots of GP130 D1 Domain. To improve binding affinity, we applied MLSD to dock two drug scaffolds, one from pool 1 and the other from pool 2, to the two binding hot spots of GP130 in order to effectively disrupt multiple key residues of IL-6. Table 1 lists the top sets of drug scaffolds binding to the hot spots of the GP130 D1 domain from MLSD docking screening. Figure 4A illustrates the binding modeling of top fragments to the hot spots of GP130.

Table 1. Predicted Docking Energies (ΔE) of the Top Combinations of Drug Scaffolds to the Hot Spots of GP130 D1 Domain

scaffold set	docking ΔE (kcal/mol)	binding modes (mimicking hot spots)
S1 (fb1, f2)	−9.4	Leu57 (fb1), Trp157 site, and extra pocket (f2)
S2 (fb1, f5)	−9.5	Leu57 (fb1), Trp157 site, and extra pocket (f5)
S3 (fb2, f5)	−9.3	Leu57 (fb2), Trp157 site, and extra pocket (f5)
S4 (fb2, f2)	−9.2	Leu57 (fb2), Trp157 site, and extra pocket (f2)

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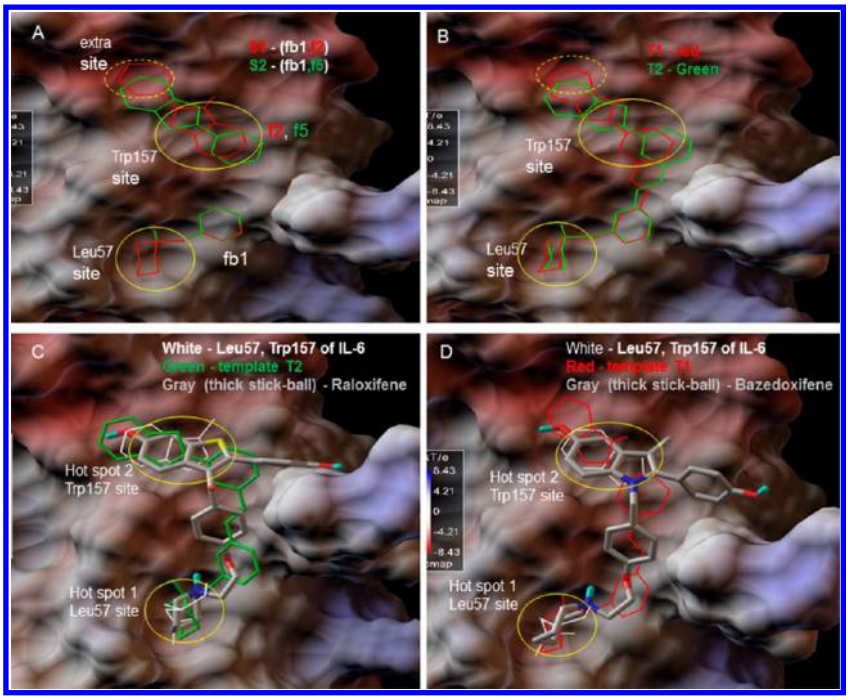

Figure 4. Docking modeling of the best drug scaffolds, the virtual templates, and drug candidates to GP130. (A) In top hits of fragment sets S1 (red) and S2 (green color), fragment fb1 (phenylpiperdinyl) occupied the Leu57 hot spot while f2 (2-phenylindole) and f5 (2-phenyl-1-benzothiophene) bound to the Trp157 binding site of GP130 with binding energies of −9.4 and −9.5 kcal/mol, respectively. (B) Tethering fragments in hits S1 and S2 gave virtual template compounds T1 (red) and T2 (green), respectively. Docking free energies and binding modes of template compounds T1 (−9.1 kcal/mol) and T2 (−9.3 kcal/mol) are close to the corresponding fragments in hits S1 and S2. (C) Drug raloxifene was identified as a novel inhibitor of GP130 D1 by similarity searching for T2 in DrugBank. Docking of raloxifene (thick gray ball-and-stick) to GP130 D1 showed that piperidinyl and 1-benzothiophene moieties mimic the native Leu57 and Trp157 (in white lines) of IL-6 in the hot spots, respectively. (D) Docking of drug candidate bazedoxifene (thick gray ball-and-stick) to GP130 D1 domain. The indole moiety and seven-membered ring azepanyl of bazedoxifene almost overlap with the native Trp157 and Leu57 residues (white lines) of IL-6, respectively.

Linking Fragments for Hits and Designing Virtual Template Compounds. To obtain possible virtual lead compounds, we linked the two fragments in hits S1 and S2 using different chemical linkers. Table 2 lists structures and docking binding energies of the top two template compounds T1 and T2. Figure 4B shows the docking simulation of T1 and T2 to GP130 D1 domain.

Drug Repositioning: Raloxifene and Bazedoxifene Identified as Potential Novel GP130 Inhibitors. The docked fragments define a blueprint for possible binders. We performed structure similarity searches of the top fragments or virtual template compounds on DrugBank database to identify FDA approved drugs or experimental drugs with a certain degree of similarity (Tanimoto similarity coefficient of $\geq$0.5).[19,21] Table 3 lists the top hits ranked by docking energy (ΔE) and visual inspection of binding modes. Existing drugs raloxifene and bazedoxifene were identified as the top two hits. The rest of the candidates in the list are experimental drugs. Raloxifene matched T2 virtual compounds as a top hit. Interestingly, similarity or substructure search of the best two fragments S2 (fb1, f5) on drug database could also identify raloxifene as a drug candidate in this case. Bazedoxifene was identified because it is a close analogue of hit DB07991, which has a structure similar to the template compound T1. Figure 4C and Figure 4D show the docking of raloxifene and bazedoxifene to GP130 D1 domain. Both raloxifene and bazedoxifene are known as selective estrogen receptor modulators (SERMs) and commonly used as treatment for osteoporosis.

Docking Modeling of the Best Drug Scaffolds, the Virtual Template, Aand Drug Candidates to GP130. Figure 4A shows the top fragment sets S1 (fb1 and f2, red) and S2 (fb1 and f5, green) docked to the hot spots of the GP130 D1 domain, with similar binding energies. In both hits, the aromatic fragment f2 (2-phenylindole in S1) or f5 (2-phenyl-1-benzothiophene in S2) occupied the Trp157 subpocket and partially occupied the extra pocket. In addition, the indole moiety of f2 and benzothiophene of f5 had aromatic interactions with Tyr94 of GP130. The hydrophobic fragment fb1 (phenylpiperdinyl) occupied the main Leu57 binding pocket formed by surrounding residues Cys6, Phe36, Ile83, and Val94. Also piperdinyl formed a hydrogen bond with the backbone of Asn92. Further binding mode clustering analysis revealed that with similar binding energies, the binding modes of f2, f5, and fb1 could be dynamic and slightly different in the hot spots. These docking results suggest that indole and benzothiophene are privileged scaffolds mimicking the Trp157 residue of IL-6, and the piperdinyl can be a more effective replacement of the flexible aliphatic tail for displacing the Leu57 residue.

Figure 4B illustrates docking of the linked template compounds T1 and T2 to the GP130 D1 domain. The top hit T2 had binding modes and binding energy (ΔE of −9.3 kcal/mol) very close to that of the fragments fb1 and f5 in hit S2. Hit T1 also bound well to Leu57 and Trp157 binding sites, as well as part of the extra subpocket, with a binding energy ΔE of −9.1 kcal/mol. They have better docking binding energies and binding modes than MDL-A. Here the virtual compounds designed have much simpler structures than the MDL-A and its

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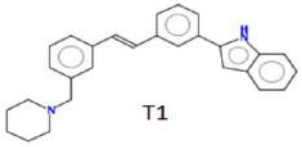
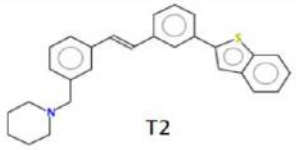
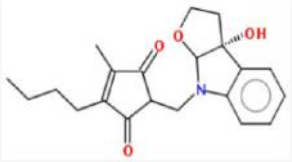
Table 2. Structures and Docking Energies (ΔE) of Template Compounds Obtained by Linking Fragments in Hits S1 (fb1 and f2) and S2 (fb1 and f5)

Compound	Structure	Predicted binding energy ΔE(kcal/mol)	Binding sites
T1		-9.1	Leu57, Trp157 and extra subpocket
T2		-9.3	Leu57, Trp157 and extra subpocket
MDL-A [a]		-6.6	Leu57, Trp157

[a]Inhibitor MDL-A is listed here for comparison.[7]

analogues and could be easier to synthesize. In addition, the templates are made of real drug scaffolds, which could potentially maintain their good drug property profiles.

Figure 4C shows docking of drug candidate raloxifene (gray ball-and-stick) to the GP130 D1 domain. Compared with the native binding of IL-6, the piperidinyl and aromatic 1-benzothiophene fragments of raloxifene almost overlapped with that of the native hot spot residues Leu57 and Trp157 (shown in white lines) of IL-6, respectively. Figure 5 shows the detailed binding interactions of raloxifene with the key active site residues of GP130 D1 domain. Asn92 and Cys6 formed two hydrogen bonds with the N and O of the piperidinylethoxy moiety of raloxifene, respectively. The binding modeling suggests that raloxifene could effectively disrupt the native IL-6 binding interaction with the GP130 D1 domain and thus block the homodimerization of GP130. Raloxifene has better binding modes and significantly improved binding energy (ΔE = −8.8 kcal/mol) than the known inhibitor MDL-A (ΔE = −6.6 kcal/mol).

Figure 4D depicts that bazedoxifene (gray ball-and-stick) has similar binding modes compared with raloxifene. The docking simulation shows that the indole fragment of bazedoxifene superimposed almost perfectly with the native Trp157 residue of IL-6 in hot spot 2. The azepanyl, a seven-membered heterocyclic tertiary amine, could effectively displace the Leu57 of IL-6 in binding hot spot 1 of the GP130 D1 domain to disable IL-6/GP130 D1 interactions. The binding mode of bazedoxifene was similar to that of template T1 (red line).

Detection of the Binding of Raloxifene and Bazedoxifene to GP130 by Drug Affinity Responsive Target Stability (DARTS) Assay. DARTS is a general method for studying the specific protein−ligand binding interactions.[21−23] This method is based on the principle that the target protein structure might be stabilized and become less susceptible to proteolysis by proteases upon drug binding. DARTS assay can be performed using complex protein mixtures such as cell lysates without the need of purified proteins. In a recent report, this method was successfully used to assess the direct binding of potential inhibitor SC144 to target GP130 in human ovarian cells.[24] To investigate the binding of drug candidates to GP130, we performed DARTS assays using human RH30 sarcoma cell lysates treated with raloxifene and bazedoxifene separately, following the protocol as previously described.[21−24] As seen in Figure 6, we found an increase in abundance of GP130 band with increasing dose of raloxifene or bazedoxifene, which demonstrates that target GP130 was protected from proteolysis upon drug binding. Particularly, in the presence of raloxifene or bazedoxifene (100 and 1000 μM), the abundance of GP130 increased significantly compared with the no drug control. These results show the direct binding of raloxifene or bazedoxifene to GP130 and suggest that the binding interactions may induce structure changes in GP130 and cause differential proteolysis by pronase.

Raloxifene Selectively Down-Regulates the IL-6 Induced STAT3 Phosphorylation in a Dose-Dependent Manner. Raloxifene is commonly known as a selective estrogen receptor α (ERα) inhibitor. To exclude interference of ER, human pancreatic cancer cell line (PANC-1) that expresses GP130, but not ER, was used in our study. To examine the inhibition of IL-6 induced downstream STAT3

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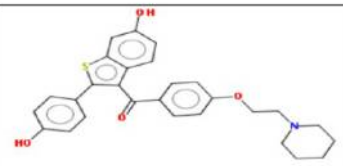
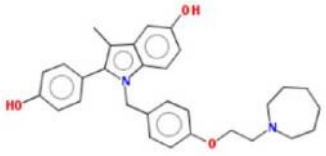
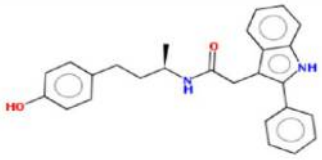
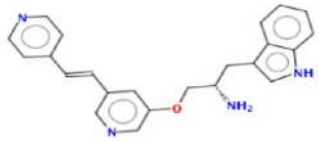
Table 3. Structures and Docking Energies of Top Hits Identified by Drug Repositioning Search with Virtual Template Compounds in Table 2

Compund	Structure	Docking ΔE (-kcal/mol)	Binding site
raloxifene		-8.8	Leu57, Trp157
bazedoxifene		-8.2	Leu57, Trp157
DB07991		-7.4	Leu57, Trp157
DB07107		-8.2	Leu57, Trp157

phosphorylation, we performed Western blot assays to detect the amount of phosphorylated STAT3 (P-STAT3) after PANC-1 cells were incubated with IL-6 and treated with raloxifene (10−50 μM).[28−30] As shown in Figure 7A, the amount of P-STAT3 stimulated by IL-6 in PANC-1 cells decreased with increasing doses of compound raloxifene. Significant reduction of phosphorylation of STAT3 induced by IL-6 was observed by treatment of 25 μM raloxifene. By contrast, raloxifene showed little inhibition on STAT3 phosphorylation induced by leukemia inhibitory factor (LIF). Also raloxifene had little inhibitory effect on STAT1 phosphorylation induced by IFN-γ. Previous reports have found that IL-6 induces the formation of IL-6/IL-6Rα/GP130 heterotrimer. Further reciprocal interactions of IL-6 and the GP130 D1 domain of the two IL-6/IL-6Rα/GP130 trimers led to homodimerization of GP130 into the hexamer via D1 domain, which triggers downstream STAT3 phosphorylation.[2,3] In contrast, LIF activates the formation of LIF/LIFR/GP130 heterotrimer for STAT3 phosphorylation, which does not require the D1 domain of GP130 to form a hexamer for signaling.[25,26,31] Mechanistically, raloxifene could most likely disrupt the IL-6/GP130 D1 interface to block the homodimerization of GP130 into hexamer, resulting in inhibition of the downstream STAT3 phosphorylation. The reasons are threefold: (1) DARTS assay proves that raloxifene binds to GP130; (2) IL-6 signaling requires D1/D2/D3 domains, and LIF signaling requires only D2/D3 domains; (3) raloxifene only disrupts IL-6 signaling but not LIF signaling. Our finding also supports the previously proposed mechanism of the known inhibitor MDL-A, which down-regulates IL-6 activity through prevention of homodimerization of GP130 to form the hexamer.[7] Raloxifene appears to be more potent than the natural product compound MDL-A, which is consistent with our binding modeling results.

Bazedoxifene Selectively Inhibited STAT3 Phosphorylation (Y705) Stimulated by IL-6. We also investigated whether bazedoxifene could inhibit STAT3 phosphorylation induced by IL-6/GP130 dimerization in human pancreatic cancer cell line (PANC-1). Following the same protocol, a Western blot was performed to detect the amount of phosphorylated STAT3 (P-STAT3) after PANC-1 cells were incubated with 50 ng/mL IL-6 or IFN-γ for 30 min, followed by the treatment of bazedoxifene at 15 or 25 μM.[14,29] Similar to the results of raloxifene, Western blot assay showed that bazedoxifene inhibited the STAT3 phosphorylation (P-STAT3) induced by IL-6 but had little effect on STAT3

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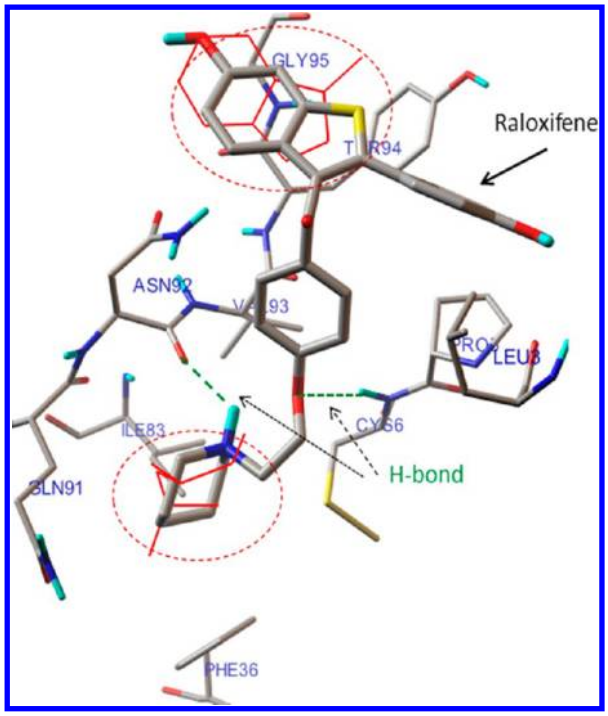

Figure 5. Modeling of binding interactions of raloxifene with the key residues in the active site in the GP130 D1 domain. Raloxifene is in thick ball-and-stick representation. Native residues Leu57 and Trp157 of IL-6 are thin red lines. Residues of GP130 are labeled in blue color. Asn92 and Cys6 form hydrogen bonds (green dotted lines) with raloxifene.

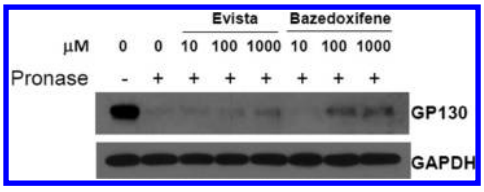

Figure 6. DARTS assay to estimate raloxifene and bazedoxifene binding to target GP130. In RH30 sarcoma cell lysates, the proteolysis of GP130 by pronase is decreased by the treatment of raloxifene (Evista) or bazedoxifene (100 and 1000 μM). The results suggest that the direct binding of raloxifene or bazedoxifene to GP130 stabilizes the structure of GP130 and therefore protects it from proteolytic digestion.

phosphorylation induced by LIF and on STAT1 phosphorylation induced by IFN-γ (Figure 7B). These results suggest that bazedoxifene could possibly disrupt the IL-6/GP130 D1 domain interface that triggers the homodimerization of GP130 and the downstream STAT3 phosphorylation, similar to raloxifene.

Assay on Constitutive STAT3 Activity Level in Cancer Cell Lines with Treatment of Raloxifene, Bazedoxifene, and MDL-A. We investigated the inhibitory effects of raloxifene and bazedoxifene on the constitutive STAT3 activity level with human breast cancer cells (SUM159) that express elevated levels of IL-6 but not ER. Following the same protocol as described before, we performed Western blot assays to detect the inhibition of persistent STAT3 phosphorylation (P-STAT3) level after SUM159 cells were treated with raloxifene (15 or 50 μM) or bazedoxifene (10–30 μM) separately. Figure 8 shows that both raloxifene and bazedoxifene significantly inhibited constitutive STAT3 activity (P-STAT3) induced by IL-6 and GP130 in SUM159 cancer cells.

In STAT3 signaling pathway, the constitutive STAT3 phosphorylation activity triggers a cascade of dimerization, nuclear translocation, and DNA binding to stimulate cancer cell proliferation. Furthermore, we performed cancer cell viability assays using the same SUM159 cancer cell culture to examine the inhibitory effects of raloxifene, bazedoxifene, and MDL-A. Table 4 shows that raloxifene, bazedoxifene, and MDL-A inhibited the cancer cell proliferation in the presence of IL-6. IC_{50} values are as follows: raloxifene, 43.2 μM; bazedoxifene, 24.9 μM; MDL-A, >250 μM.

Since SUM159 does not express ER, it is clear that the inhibitions of constitutive P-STAT3 level and cell proliferation by these drug candidates are not through modulation of the ER target. Previous studies indicate that IL-6/GP130/STAT3 is one of the major cancer proliferation pathways. Our finding that raloxifene and bazedoxifene inhibit the constitutive P-STAT3 activity and downstream cell proliferation in the presence of IL-6 and GP130 would provide supportive evidence that the inhibition might be due to attenuation of IL-6/GP130/STAT3 signaling. In our computational simulation, binding hot spots of raloxifene and bazedoxifene are the same as the known IL-6/GP130 inhibitor MDL-A, which could further support our hypothesis.

IL-6 is a cytokine involved in initialization and maintenance of inflammatory, autoimmune response, osteoporosis, and proliferation. Raloxifene is commonly used as a selective estrogen receptor modulator (SERM) for treatment of osteoporosis. However, several reports also demonstrated that raloxifene can modulate osteoclast activity by, at least in part, an IL-6 and TNF-α dependent mechanism. Raloxifene can simultaneously stimulate osteoprotegerin and inhibit IL-6 production in human trabecular osteoblasts.[32−35] IL-6 may also be a key factor associated with osteoporosis in patients with rheumatoid arthritis (RA). In a mouse model of RA, raloxifene decreased the IL-6 levels and protected both RA and the associated osteoporosis.[35−38] The mechanism of IL-6 inhibition by raloxifene is not clear. Raloxifene could directly inhibit IL-6 expression or alternatively inhibit the IL-6/GP130 interface; both mechanisms could down-regulate the IL-6/GP130/STAT3 pathway. It is interesting that the known IL-6/GP130 inhibitor MDL-A also prevents the bone resorption and loss by a mechanism different from that of 17β-estradiol acting on ER in an experimental model of postmenopausal osteoporosis in vivo.[7] These findings suggest that modulation of cytokine IL-6 by raloxifene might be an alternative and secondary mechanism of osteoporosis prevention. One of the off-target effects of raloxifene could be inhibiting IL-6 related signaling pathways, one of which could be the IL6/GP130/STAT3 crosstalk signaling pathway for cancer. We attempted to repurpose raloxifene to target IL-6/GP130/STAT3 signaling pathway of cancer cells. Cancer cell line assays provide indirect, yet supportive evidence for the proposed mechanism and suggest it seems likely that raloxifene or bazedoxifene selectively disrupts the IL-6/GP130 interface and the homodimerization to inhibit constitutive P-STAT3 activity stimulated by IL-6.

■ CONCLUSIONS

It is challenging to design small molecules to disrupt multiple binding "hot spots" on the interface of protein−protein interaction. Our MLSD method could serve to dock multiple

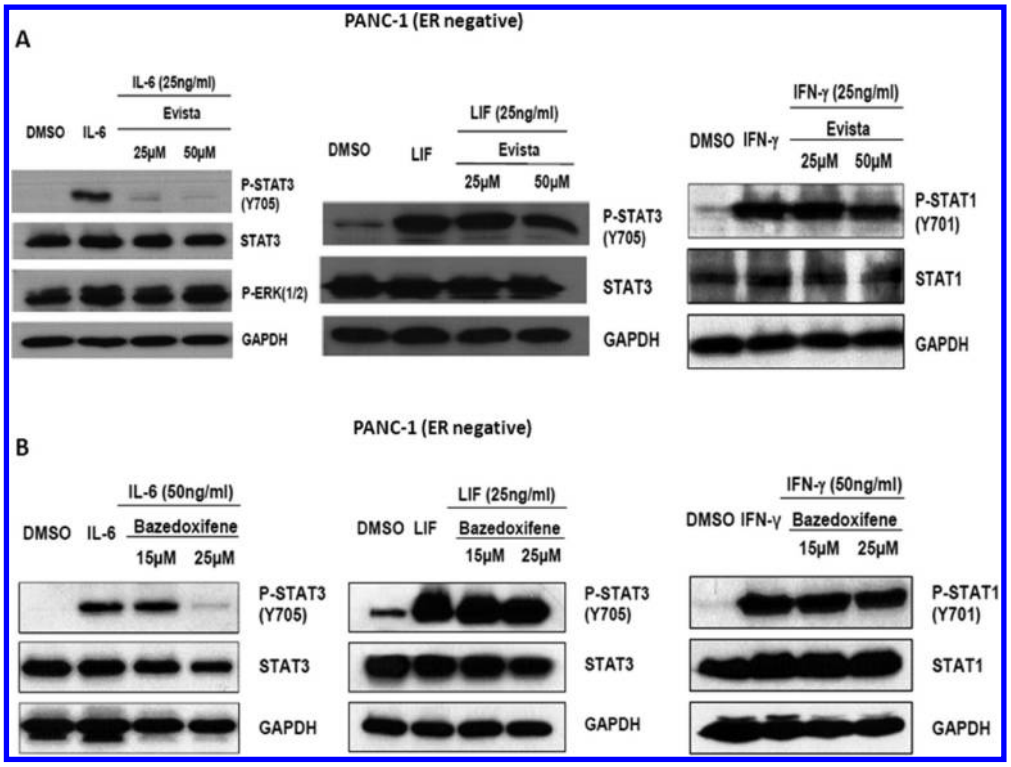

Figure 7. Selective inhibition of the IL-6 induced P-STAT3 by raloxifene (Evista) and bazedoxifene. (A) In human pancreatic cancer cells (PANC-1, ER negative) with treatment of IL-6 and raloxifene (25 μM), the downstream STAT3 phosphorylation (P-STAT3 at Y705) stimulated by IL-6 was significantly inhibited. In contrast, the P-STAT3 induced by LIF or P-STAT1 induced by IFN-γ was not suppressed. (B) Bazedoxifene (25 μM) selectively inhibited P-STAT3 induced by IL-6 but not P-STAT3 induced by LIF or P-STAT1 induced by IFN-γ.

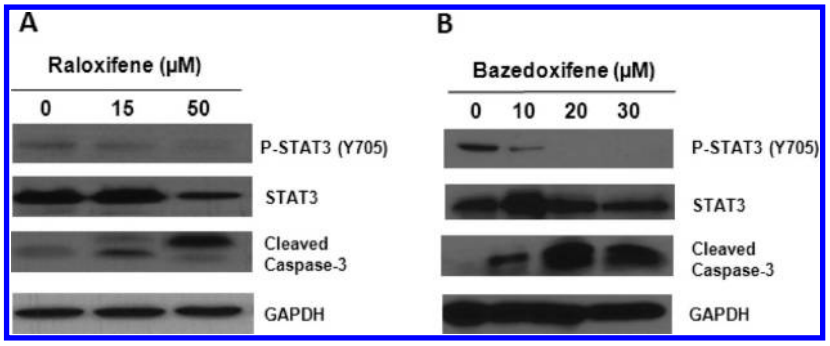

Figure 8. Effect of raloxifene and bazedoxifene on constitutive STAT3 activity level in SUM159 cancer cells. (A) In human breast cancer cells (SUM159, ER negative) expressing IL-6 and GP130, raloxifene (15−50 μM) down-regulated the constitutive STAT3 phosphorylation (P-STAT3) activity level. (B) Bazedoxifene (10−30 μM) inhibited the constitutive P-STAT3 activity levels induced by the expressed IL-6 and GP130.

Table 4. Docked Binding Energies and IC_{50} Values of Raloxifene, Bazedoxifene, and MDL-A in SUM159 Cell Viability Assays

	IC_{50} (μM)	
compd	docking energy ΔE (kcal/mol)	SUM159 (ER negative)
raloxifene	−8.8	43.2
bazedoxifene	−8.2	24.9
MDL-A	−6.6	>250.0

fragments to these binding hot spots on the interface to simultaneously interfere with the binding of multiple key residues among the protein binding partners. In this work, the combined MLSD and drug repositioning approach quickly identified that raloxifene and bazedoxifene, two known selective estrogen receptor modulator drugs for osteoporosis, show potential for disrupting IL-6/GP130/STAT3 cancer signaling pathway through disruption of the IL-6/GP130 D1 interface. They represent a new class of lead compounds with piperidine, benzothiophene, and indole scaffolds to inhibit homodimerization of GP130. Thus, the discovery of raloxifene and bazedoxifene could speed up the development of clinical therapies for the IL-6/GP130/STAT3 dependent cancers. And of course, they can also be used as leads for further optimization for IL-6 signaling inhibition. Our approach to combine multiple fragment simultaneous docking and drug repositioning could potentially help to design novel inhibitors targeting other protein−protein interactions.

■ EXPERIMENTAL SECTION

All chemical reagents and solvents were purchased from commercial sources and used without further purification. Compounds raloxifene·

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HCl and bazedoxifene·HCl were purchased from Selleckchem with 99.8% purity. Other compounds have ≥95% purity as determined by HPLC.

Privileged Drug Scaffolds Preparation for GP130. To prepare the drug scaffold library for GP130, the known inhibitors MDL-A and its analogues were used to generate a set of feature fragments for the binding hot spots of the D1 domain of GP130. These known inhibitors were fragmented by a retro-synthetic approach. In addtion, the key interacting residues Leu57 and Trp157 of IL-6 were included as feature fragments. Two databases were used to identify drug scaffolds for binding to the GP130 D1 domain. One was the top drug scaffold database generated from FDA approved drugs (AD) and extended drugs (Ed) as reported by Wang et al.[18] The other database was DrugBank, which contains 6707 drug entries including 1436 FDA approved small molecule drugs, and 5086 experimental drugs.[19] Drug scaffolds for GP130 were then identified by structure or chemical feature search of the obtained feature fragments on top drug scaffolds library and by substructure search on DrugBank. The chemical features of a fragment include aromatic ring, polarity, hydrogen bonding, and hydrophobicity. The privileged drug scaffolds library identified for the GP130 D1 domain was used for multifragment docking screening (Figure 3B).

Multiple Fragment Simultaneous Docking Screening and Drug Repositioning. The crystal structure of the GP130 D1 domain (PDB code 1P9M) was used as the receptor for docking. MLSD docking screening and drug repositioning were employed as previously described.[13,14,27] Privileged drug scaffolds in Figure 3B were used as fragments in MLSD screening. In multifragment docking, two fragments from the two pools of the drug scaffolds were used to probe binding subpockets of the GP130 D1 domain. Systematic multifragment docking screening with the combinations of drug scaffolds was ranked by the predicted binding energy. The docked fragments with a predicted binding energy of <−7.0 kcal/mol (better than MDL-A docking energy of −6.6 kcal/mol) were considered for further visual inspection of binding modes analysis. A set of fragments with good binding energies and binding modes were selected as hits. Previously reported procedure and parameter settings were used for multiple fragments simultaneous docking.[13] Hybrid particle swarm optimization (PSO) or Lamarckian genetic algorithm (LGA) was used as a searching method in MLSD docking screening. A time decreasing inertia weighted PSO (w from 0.9 to 0.4) algorithm was used. The cognitive (c_1) and social (c_2) acceleration factors of PSO were set as $c_1 = c_2 = 2.0$. Virtual template compounds were obtained by linking docked fragments using various types of tethers. The candidates were optimized for the two major drug properties: octanol−water partition coefficient (log P) and polar surface area (PSA). Chemical 2D structure and 3D structures were generated by Molinspiration WebME Editor and Web service. Drug properties were calculated using Molinspiration molecular property service (http://www.molinspiration.com). The linked compounds were re-docked to the GP130 D1 domain and ranked by binding energies and binding mode closeness to its corresponding fragments docked. To apply the drug repositioning concept, similarity searches for virtual compound hits in DrugBank (http://www.drugbank.ca/) and PubChem (http://pubchem.ncbi.nlm.nih.gov/) databases were performed to identify potential drug analogues of hit compounds.[19,20] SMILES encoding and Tanimoto similarity coefficient cutoff of 0.5 were used for similarity search. The identified hit compounds were verified by computational re-docking using MLSD and ranked by predicted binding energies and visual inspection of binding modes before selection for purchase to perform cell line assays.[27] The MLSD program and source code are available for free upon request.

Cancer Cell Assays. *Cell Lines and Cell Culture.* Human pancreatic cancer cell (PANC-1) and human breast cancer cell (SUM159, ER negative) lines were purchased from the American Type Culture Collection (ATCC). Cancer cell lines were cultured in DMEM medium supplemented with 10% FBS and 100 U/mL penicillin/streptomycin B, in a humidified 37 °C incubator with 5% CO_2.

Western Blot Analysis. PANC-1 and SUM159 cells were treated with raloxifene (10−50 μM), bazedoxifene (10−30 μM), or DMSO control at 60−80% confluence in the presence of 10% fetal bovine serum (FBS) for 24 h and lysed in cold RIPA lysis buffer containing a cocktail of protease inhibitors to prepare whole-cell extracts.[14,28,29] Lysates were then centrifuged at 14 000 rpm for 10 min to remove insoluble materials. Then 30−100 protein samples were separated by SDS−PAGE and transferred onto a PVDF membrane. After being blocked with 5% nonfat milk, the proteins were immunoblotted overnight at 4 °C with 1:1000 dilution of primary antibodies (Cell Signaling Technology) against phospho-STAT3 (pTyr705), STAT3, phospho-STAT1 (tyrosine 701), STAT1, and GAPDH, respectively, and 1:10,000 dilution of HRP conjugated secondary antibody for 1 h at room temperature. The target proteins were visualized by chemiluminescence (Cell Signaling Technology).

DARTS Assay. The drug affinity responsive target stability (DARTS) assay was used to detect the binding of raloxifene and bazedoxifene to GP130 target by following the procedure previously described.[21−24] Human RH30 sarcoma cells were lysed using M-PER (Thermo Scientific, Inc.) supplemented with protease and phosphatase inhibitors. Lysates were then incubated with escalating concentrations (10−1000 μM) of raloxifene, bazedoxifene, or DMSO control at room temperature for 1 h. Proteolysis was followed by adding protease pronase solution at a ratio of 1 mg of pronase to 1000 mg of lysate for 30 min at room temperature. To stop proteolysis, 5× SDS sample loading buffer was added at 1:4 ratio to each sample and boiled at 100 °C. The resulted protein samples were separated by 8% SDS−PAGE gel and analyzed by Western blotting as previously described.[21,24]

Cell Viability Assay. SUM159 cells were seeded in 96-well plates at a density of 3000 cells per well. Escalating concentrations (5−100 μM) of raloxifene and bazedoxifene were added in triplicate to the plates in the presence of 10% FBS.[27−29] The cells were incubated at 37 °C for a period of 72 h. 3-(4,5-Dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) viability assay was done according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany).[30] The absorbance was read at 595 nm. Half-maximal inhibitory concentrations (IC_{50}) were determined using Sigma Plot 9.0 software (Systat Software Inc., San Jose, CA).

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

IL-6, interleukin 6; GP130, glycoprotein 130; MDL-A, madindoline A; MLSD, multiple ligand simultaneous docking; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; STAT3, signal transducer and activator of transcription 3; INF-γ, interferon γ; LIF, leukemia inhibitory factor; ER, estrogen receptor

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Design, Synthesis and Identification of Madindoline-A Analogues as Inhibitors of the IL-6/JAK/STAT Pathway

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Abstract: Madindoline A (MDL-A) is a natural product reported to inhibit the IL-6/JAK/STAT pathway through interaction with GP130 (CD130), effectively blocking the binding of the cytokine IL-6. The binding to GP130 does not prevent IL-6 from binding to the receptor complex, but rather disrupts the homodimerization of the IL-6/IL-6R/GP130 heterotrimeric complex, resulting in inactivation of the pathway. The exact binding site of madindoline A to GP130, however, is unknown. A molecular model of the receptor complex was created and a docking study carried out with GP130 to determine the binding site of the natural product. Using this data and a fragment-based design approach, two analogues of madindoline A (**1** and **2**) were designed and synthesized which demonstrate increased potency relative to the natural compound with respect to GP130 binding affinity. Inhibition of STAT3 phosphorylation in MCF-7 breast cancer cells stimulated with IL-6 and SUM159 breast tumor both *in vitro* and *in vivo* show great potential for novel breast cancer therapy with these compounds..

Interleukin-6 (IL-6) is a multifunctional cytokine in the family of GP130 cytokines which plays a key role in cellular functions which include immune response, cell survival, apoptosis, metastasis, and cell proliferation, suggesting the potential importance of IL-6 in inflammatory disease and cancer.{Kishimoto, 2005 #2} With regard to cancer, although IL-6 has been shown to have both tumor-promoting and inhibiting effects, elevated serum levels of IL-6 have been associated with a poor prognosis in several cancer types including breast and prostate cancers.{Culig, 2005 #13}{Salgado, 2003 #50} IL-6 is responsible for growth stimulation or regulation in many types of cancer cells through the induction of various signaling pathways, including the critical Janus kinase/Signal Transducers and Activators of Transcription (JAK/STAT) pathway. The JAK2/STAT3 pathway mediates gene transcription and thereby directly influences growth, differentiation, and apoptosis in the cancer cells.{Schindler, 1995 #43} Mounting evidence in numerous cancer types indicates the importance of STAT3 in cancer progression and its dependence on IL-6.{Culig, 2005 #13} IL-6 initiates the JAK2/STAT3 signaling cascade via interaction with the extracellular domains of IL6-R and GP130 via a heterodimeric IL-6/IL-6R/GP130 complex. This dimerization event triggers the activation of JAK, which subsequently phosphorylates

Oral Presentation at the 2011 Era of Hope Conference

Abstract

Novel IL-6 inhibitors for Breast Cancer Therapy

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The Purpose

Increasing evidence points to IL-6 as a new anti-cancer target. Recent research shows that (1) IL-6 is a potential regulator of breast tumor stem cell self-renewal, implicating IL-6 as a critical factor in tumor mammosphere revival and resistance. (2) In invasive breast tumors, the percentage of cases showing immunoreactivity for IL-6, gp130, and IL-6Rα was much higher than in non-malignant lesions. A systematic review establishes IL-6 as a clear negative prognosticator, especially in breast metastatic and recurrent cases. However, currently there is neither drug nor drug research directed toward this new exciting target. Our strategy is to use structure-based computational approach to re-engineer a natural product compound, MDL-A, to make more potent and specific, synthetically tractable small molecules to disrupt the IL-6/GP130 interaction, thus inhibiting the GP130 functional dimerization and ensuing STAT3 activation. The ultimate aim is to create a new therapeutic direction to target tumor microenvironment and to disable the IL-6/IL-6R/GP130 oncogenic hexameric complex, leading to a new way to overcome breast drug resistance and breast cancer recurrence.

The Results

A systematic structure-based computer-aided molecular design has been done, pointing to compounds with high potentials to be developed into nontoxic, orally available drug without worrying about blood serum binding. Currently two generations of inhibitors have been synthesized and tested. MDL-5, one of the 1st generation inhibitors with the natural compound pentendione ring replaced by hydroxylbenzene and an additional carboxylphenyl ring identified through fragment-based design, shows reduced STAT3 phosphorylation/activation with IC_{50} of 5μM and 20μM on MCF-7 (with elevated IL-6 expression) and MDA-MB-453 (upon IL-6 stimulation) breast cancer cells, respectively. MDL-16, one of the 2nd generation inhibitors with an indolino fragment serving as core scaffold built upon MDL-5, improves the potency to sub-micro IC_{50} for MCF-7 breast cancer cells. Both compounds do not induce cell death in human normal PBMCs. More testings on the other breast cancer cell lines and normal cells from the bone marrow and intestinal tract are on-going. In addition, *in vivo* experiments are planned for the next three months. The results so far are very promising, pointing to exciting potential novel breast cancer therapeutic option through IL-6 inhibition, especially for breast metastatic and recurrent cases.

Disruption of IL-6 signaling in the IL-6/JAK/STAT pathway using small molecules

By: Fuchs, James R.; Jena, Nivedita; Kumari, Vandana; Mok, May; Li, Pui-Kai; Li, Chenglong

Abstracts of Papers, 242nd ACS National Meeting&Exposition, Denver, CO, United States, August 28-September 1, 2011

Interleukin-6 (IL-6) is a multifunctional cytokine which displays both tumor-promoting and inhibiting effects. IL-6 is responsible for growth stimulation in many cancer cells through the induction of various signaling pathways, including the JAK/STAT pathway, which mediates gene transcription and thereby influences growth, differentiation, and apoptosis in cancer cells. This cascade is initiated through interaction of IL-6 with the extracellular domains of IL6-R and GP130 and subsequent dimerization of this trimeric complex. In 1996, madindoline A, a natural product, was reported to suppress the IL-6/JAK /STAT signaling cascade via binding to GP130, resulting in inhibition of the homodimerization event. Using this natural product as a starting point, we applied computational and synthetic techniques to develop a series of compds. capable of preventing STAT3 phosphorylation via disruption of IL-6 signaling. These studies have identified several promising compds. with greater biol. activity than madindoline itself and provide strategies for addnl. structural modification.

Small molecules targeting IL-6/GP130 homodimerization in the IL-6/JAK/STAT pathway

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Abstracts of Papers, 243rd ACS National Meeting&Exposition, San Diego, CA, United States, March 25-29, 2012

The IL-6/JAK/STAT pathway is a key signal transduction pathway activated in many cancers which results in the translocation of phosphorylated STAT dimers to the nucleus where they regulate gene transcription. The process is activated by interleukin-6 (IL-6) through an IL-6/GP130 homodimerization event. Inhibition of this homodimerization and subsequent disruption of downstream phosphorylation would provide a new target for cancer therapy. Therefore, two series of compds. (indoline- and indole-contg. scaffolds) were initially designed and synthesized based on the structure of madindoline A, a weak inhibitor of IL-6. Mol. modeling suggests their binding to the D1 domain of GP130, thereby preventing interaction with IL-6. These results are supported through binding studies with the GP130 protein and obsd. inhibition of pSTAT3. Subsequent modification has led to addnl. compds. which effectively inhibit the pathway. The design, synthesis, and biol. evaluation of these analogs in various cell lines will be reported and discussed.

List of personnel receiving pay from the research effort:

- Chenglong Li, PI
- Vandana Kumari (graduate student, Li Lab)
- Min Wang (postdoc, Li Lab)
- James Fuchs, co-I
- Chido Hambira (graduate student, Fuchs Lab)
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